NEW SOURCES OF SALT TOLERANCE IN DURUM WHEAT

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February 2007

A thesis submitted for the degree of Doctor of Philosophy at the Australian National University

DECLARATION OF ORIGINALITY

This thesis reports the original work of the author. The experiments and the written text are those of the author.

Chapter 2 – This chapter was published as part of the following publication:

Munns R, James RA (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant and Soil* **253**, 201-218.

Chapter 3 – This chapter was published as:

James RA, Rivelli AR, Munns R, von Caemmerer S (2002) Factors affecting CO_2 assimilation, leaf injury and growth in salt-stressed durum wheat. *Functional Plant Biology* **29**, 1393-1403.

Chapter 4 – This chapter was published as:

James RA, Munns R, von Caemmerer S, Trejo C, Miller C, Condon AG (2006) Photosynthetic capacity is related to the cellular and subcellular partitioning of Na^+, K^+ and Cl^- in salt-affected barley and durum wheat. *Plant, Cell and Environment* **29**, 2185-2197.

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February, 2007

ACKNOWLEDGEMENTS

I gratefully acknowledge CSIRO Plant Industry for awarding me a part-time CSIRO Postgraduate Studentship to undertake PhD studies. Special thanks to Drs Richard Richards and Rana Munns for supporting my application, and therefore making the PhD possible.

I am deeply indebted to my main supervisor Dr Rana Munns. Thank you Rana for your friendship and support over many years and particularly, for your patience, guidance and direction over the course of my PhD research. I also thank my co-supervisors, Drs Susanne von Caemmerer and Tony Condon, for excellent guidance in experimental setup, for sharing their expertise in various techniques and also for critical comments on the thesis.

I would like to thank Dr Ray Hare for selecting and providing me with the tetraploid landraces used in Chapter 2, and for his support and friendship. I thank the Analytical Services team at PI, and particularly Lorraine Mason and Richard Philips for atomic absorption spectroscopy, and Trish Wallace for XRF analysis. Their prompt and careful analysis of leaf samples was always appreciated. I would like to thank the Microscopy Unit team at CSIRO, Plant Industry. Special thanks to Celia Miller for assistance in sample preparation and running the cryo-SEM. I also greatly appreciated advice and discussions with Drs Margaret McCully and Cheng Huang on microscopy procedures and the analysis of ions in the vacuoles of leaf cells. I thank Dr Tim Colmer (UWA) for his supervision and advice whilst I was in Perth to complete glycinebetaine analyses, and Dr Alec Zwart for assistance with statistical analysis of the conductance screens in Chapter 5. I would also like to thank Carol Blake and Karen Glover for occasional assistance with experiments and also the staff at the Potting Shed, Workshop and Phytotron for providing me with all the necessary materials for my research.

There are a number of friends and colleagues that deserve thanks. To my fellow PhD students in the 'Crèche'; Steve, Xavier, Dave and Sarah, and also colleagues past and present including Greg, John, and Vander, thanks for the assistance along the way, for friendship and encouragement and for fun exchanges that lightened the air and made the process all the more enjoyable. I would like to thank all my colleagues in the Crop Adaptation building for surrounding me with great science and wonderful synergies between disciplines that made for an exciting and productive workplace.

I would like to thank my parents, Barry and Anne James, for their wisdom and foresight in encouraging me to persevere with my education and giving me all the necessary opportunities and support. You are a great example and an inspiration to me.

I dedicate this thesis to the two special girls in my life.

To Abigail – my sweet summer breeze. You are a joy and delight. I hope that you are always amazed and enthralled at the wonder and complexity of God's wonderful creation. Lastly, and most importantly, to my beautiful wife and best friend, Maria. Thank you for your unfailing love, faithfulness, support and encouragement.

ABSTRACT

Durum (pasta) wheat (*Triticum turgidum* L. ssp *durum* Desf.) is typically very salt sensitive compared to bread wheat (*T. aestivum* L.). The development of salt-tolerant durum wheat would provide growers with an opportunity to successfully grow a premium wheat grade over wider salt-affected areas. New sources of salt exclusion have been found which could improve the adaptation of durum wheat to soils of moderate salinity. The identification of durum genotypes tolerant to high internal concentrations of Na^+ in the leaf ('tissue tolerance') and durum genotypes with osmotic stress tolerance would provide further potential for improved salt tolerance. These traits are yet to be identified in durum wheat. The aims of this thesis were: to characterise genetic variation in durum wheat and its close relatives for tissue tolerance and osmotic stress tolerance; to define physiological and biochemical mechanisms of these traits; and to evaluate the impact of these traits on plant performance.

Tissue tolerance to high internal salt concentrations was assessed in a diverse collection of various *T. turgidum* wheat genotypes. High internal Na^+ levels were identified in many landraces. Five of these landraces maintained a high percentage of green healthy leaves despite having a high Na^+ concentration in the leaves, as high as barley, indicating that they may have the ability to tolerate high Na^+ at the tissue or cellular level.

The contribution of the compatible solutes glycinebetaine and proline to osmotic adjustment and tissue tolerance was assessed. No increase in proline was detected and while significant genetic variation in glycinebetaine accumulation under salt stress was found, there was no genotypic correlation with either Na^+ accumulation or leaf injury.

To examine the physiology of tolerance to high internal salt concentrations, two *T. turgidum* genotypes that differ in the degree of salt-induced leaf injury were grown in 150 mM NaCl. A number of physiological parameters were assessed including leaf ion content, water relations, chlorophyll content, chlorophyll fluorescence and gas exchange. The growth of both genotypes was substantially reduced by salinity, but genotypic differences in growth appeared later. These differences were not related to turgor, but were associated with differences in leaf injury and with reduced CO_2 assimilation. Salinity caused a large decrease in stomatal conductance of both genotypes. Reductions in assimilation rate were initially due to lower stomatal

conductance and with time were then due to a combination of stomatal and non-stomatal limitations. The non-stomatal limitations, as indicated by chlorophyll degradation and some chlorophyll fluorescence parameters, were associated with a build up of Na^+ in the leaves above 250 mM on a tissue water basis.

A major component of tolerance of high tissue Na^+ may be the capacity to compartmentalise salt into safe storage places such as vacuoles to avoid toxic effects of salt on photosynthesis and other key metabolic processes. To test this hypothesis, the relationship between photosynthetic capacity and the cellular and subcellular distributions of Na^+, K^+ and Cl^- was studied using cryo-SEM X-ray microanalysis, in a sensitive durum wheat and a tolerant barley grown in a range of salinity treatments. Efficient cellular and sub-cellular partitioning of Na^+ and K^+ in barley led to the preservation of a favourable K^+:Na^+ ratio in the cytoplasm at higher leaf Na^+ concentrations in comparison with durum wheat. Photosynthetic capacity of barley declined at higher leaf Na^+ concentrations than in durum wheat. The maintenance of photosynthetic capacity in barley was associated with the maintenance of higher K^+, lower Na^+ and the resulting higher K^+:Na^+ in the cytoplasm of mesophyll cells.

As stomatal conductance was reduced immediately with the onset of salinity and later was the initial cause of a decline in CO_2 assimilation rate, the potential for tolerance to (salt-induced) osmotic stress in durum wheat was examined. The magnitude of the response of stomatal conductance to salt stress, before salts build up in the leaf, was used to screen a large collection of international durum varieties. Two to three-fold differences in the magnitude of the response of stomatal conductance to salt-induced osmotic stress were found. Higher stomatal conductance in salt was related to higher CO_2 assimilation rate and there was a positive relationship between stomatal conductance and relative growth rate in salt.

This study has identified potential new sources of salt tolerance in durum wheat. *T. turgidum* genotypes were identified with 'tissue tolerance', were able to maintain green leaf area and photosynthetic aspects despite high internal leaf Na^+ concentrations. Durum varieties were identified with 'osmotic stress tolerance'. These genotypes showed little stomatal closure despite the osmotic stress imposed by high salinity. Understanding physiological mechanisms at the whole plant and cellular level has been fundamental in identifying genotypic variation in these salt tolerance traits. Upon validation of these traits in the field, future work could exploit the durum and durum-

related germplasm identified with these traits, to provide novel sources of salt tolerance in a durum wheat breeding program.

DEFINITION OF TERMS

Dryland salinity:	also known as 'seepage salinity' is salinity that is associated with a rising water containing salt. Salts are further concentrated on the soil surface with high evaporation.
Transient salinity:	also known as 'subsoil salinity' is salinity that is associated with salts present in the upper soil layers, which move up and become concentrated as the soil dries.
Salt tolerance:	biomass or yield in saline relative to non-saline conditions.
Tissue tolerance:	tolerance of high leaf Na^+ concentrations, through efficient vacuolar compartmentation of salts.
Osmotic stress tolerance:	tolerance to the water stress generated by the roots encountering an osmotica (salts) in the soil solution.
Non-destructive screen:	screening method which doesn't result in the death of a plant.

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INTRODUCTION, LITERATURE REVIEW, RATIONALE AND AIMS

1. INTRODUCTION, LITERATURE REVIEW, RATIONALE AND AIMS

1.1 INTRODUCTION

1.1.1 Durum wheat production in Australia

Durum wheat (*Triticum turgidum* L. ssp. *durum* Desf.) is grown in Australia for the processing of semolina for the production of pasta. Durum flour is also used in some breakfast cereals and breads and as animal feed. In southern European and Middle Eastern countries, whole durum grains are also used for couscous, burghul and frikke.

Durum wheat production has increased in Australia since the early 1990s, peaking at about 800,000 tonnes in 2001, and averaging close to 600,000 tonnes over the last 5 years (Figure 1.1). Production was curtailed by floods in NSW in 2000 and by severe drought in 2002 and ongoing drought over the last few years (2003 – 2005).

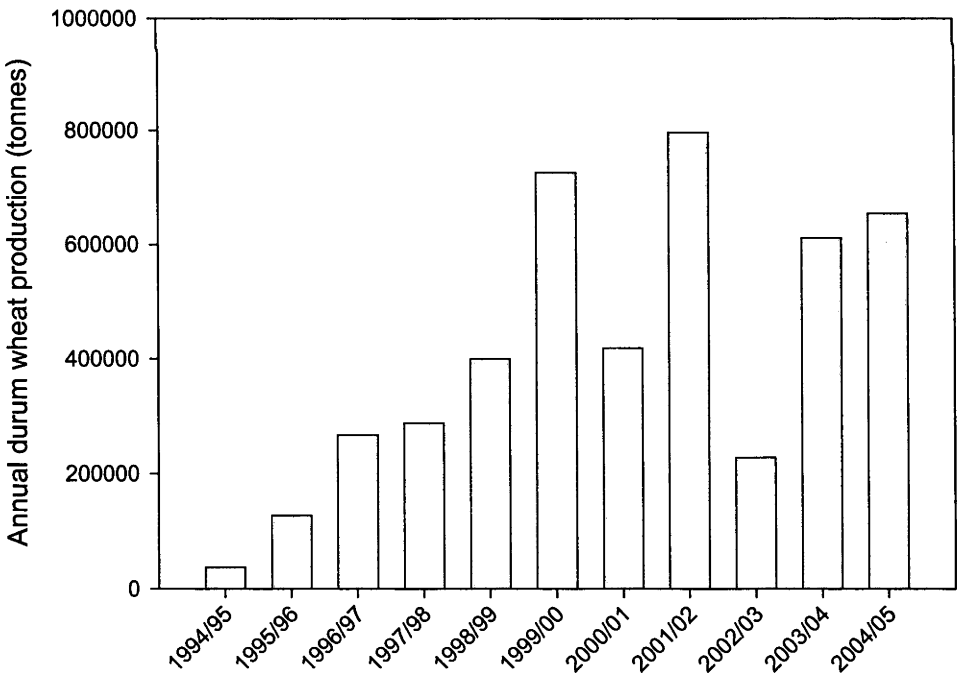

Figure 1.1 Australian durum wheat production (tonnes/year) between 1994 – 2004. Source: AWB Limited.

The established growing areas for durum wheat production in Australia are the northern NSW wheat belt and the mid-north and the Yorke Peninsula in SA. Limited production

of durum wheat commenced in WA in the mid 90s. Northern NSW produces the majority of Australian durum wheat crop, accounting for about 55%, followed by SA with about 40% (Figure1.2).

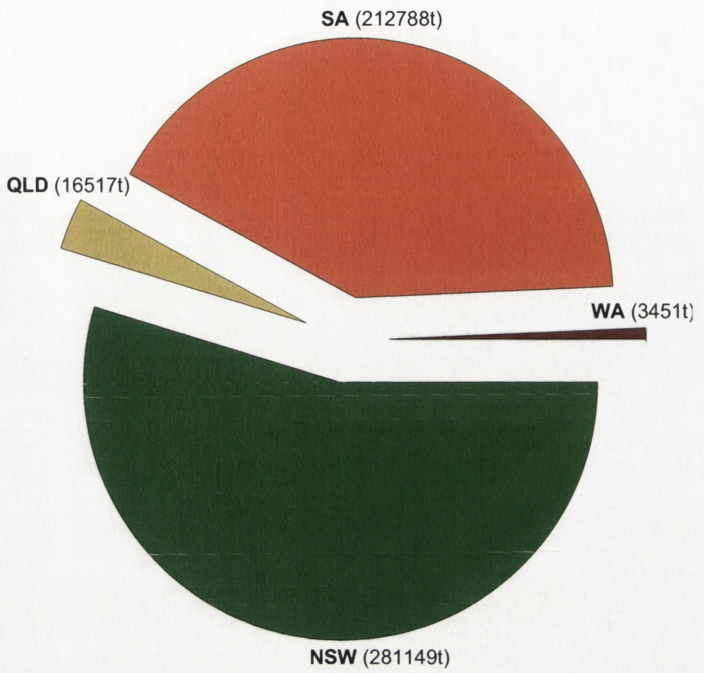

Figure 1.2 Average Australian durum wheat production (tonnes – t) by state, between 1994 – 2004. Source: AWB Limited

Durum wheat attracts a $20 - $40 per tonne premium over bread wheat, and while this premium holds on the international market, farmers want to increase production and expand the industry. Durum wheat production is already expanding into areas in southern and western Australia in which soil sodicity and salinity are prevalent. Expansion is being impeded by subsoil salinity in northern NSW and Queensland. Improving the salt tolerance of Australian durum wheat varieties will be needed to maintain profitable farming of this high-value crop in an increasingly marginal landscape and will be essential for the industry expansion to occur

1.1.2 Widespread forms of salinity in Australia

The salt in Australian soils mainly originates from that deposited by rain and wind (from the ocean) onto the Australian landscape over thousands of years and also by the weathering of rocks. This salt is distributed throughout the soil profile, largely in the groundwater in the higher rainfall areas, and in the subsoil and upper layers in the drier

areas where the combination of soil structure (e.g. clay layers) and low rainfall has restricted the leaching of salts into the groundwater.

Rengasamy (2006) has classified salinity into three broad categories:

1) Groundwater associated salinity

2) Non-groundwater associated salinity

3) Irrigation associated salinity

Groundwater associated salinity, also called 'dryland salinity' or 'seepage salinity', is a form of salinity related to a shallow or rising saline water table. Agricultural practices such as the clearing of native perennial vegetation and the utilisation of shallow-rooted winter active annual crops have contributed substantially to the rising water table. This form of salinity occurs in the higher rainfall zones of the wheat belt (450-600 mm) and is prominent in low lying areas, or discharge areas, where the water table rises to the soil surface carrying the salts in it. The salinity of groundwater can be quite high, ranging in EC (electrical conductivity) between 15 – 150 dS/m (deciSiemen/metre) (Rengasamy, 2002). The salts are further concentrated at the soil surface with high evaporation, particularly in the summer months.

Non-groundwater associated salinity, also known as 'subsoil salinity' or 'transient salinity' (Rengasamy, 2002) is associated not with a rising water table, but rather with presence of salt in the topsoil and subsoil layers. In clay soils it is associated with sodicity. Salt that is held in a seasonally saturated layer below the root zone moves up into the root zone as the soil dries, causing 'transient salinity'. This form of salinity occurs in the lower rainfall zones of the wheat belt (250-450 mm) and is much more prevalent than groundwater associated salinity. In Australia, two thirds of the agricultural area is affected by sodic soils and transient salinity, costing farmers about 1330 million dollars in lost opportunity (Rengasamy, 2002; 2006).

The third type of salinity is caused by irrigation. This form of salinity occurs through the combination of salts introduced in poor quality irrigation water, low hydraulic conductivity of sodic and clay soils and high evaporation.

1.1.3 Wheat and salinity

Wheat (*Triticum* sp.) is generally considered to be a relatively salt-tolerant species more tolerant than rice and maize, but not as tolerant as barley (Maas and Hoffman, 1977; Francios and Maas, 1994).

The major factor affecting yield of salt-stressed wheat appears to be a reduction in the number of fertile spikelets or tillers per plant. Maas and Poss (1989) found that the sensitivity of wheat decreased with plant age, thus the vegetative stage of growth (determining number of tillers and kernels per square metre) more than the reproductive and maturation stages (determining kernel weight) were affected. Additionally, El-Hendawy et al. (2005a) concluded that tiller number per plant was more affected than total leaf area by salinity and more important in determining yield under salt-stress.

Differences in salt tolerance between bread wheat and durum wheat

A number of studies in the field and in controlled environments have shown that durum wheat is more sensitive to salinity than bread wheat (Figure 1.3) and that there are probably a range of factors responsible for this sensitivity (Maas and Grieve, 1990; Shah et al., 1987; Rawson et al., 1988b; Dang et al., 2006). For example, Francois et al., (1986) found that the relative yield began to decrease at lower conductivities in the durum wheat (6 dS/m) than bread wheat (9 dS/m) and that yield reductions in the durum wheat were about 25% greater than bread wheat with increasing conductivities. Another study which examined yield parameters of bread wheat 'Probred' compared to durum wheat 'Aldura', found lower number of (fertile) tillers was the main parameter causing lower yields of salt-stressed durum wheat (Maas & Grieve, 1990). Similarly, under water-limiting conditions durum wheat was found to have poorer early vigour than bread wheat, resulting in fewer tillers/m^2 and fewer kernels/m^2 (Zubaidi et al., 1999). Interestingly even amongst durum wheat varieties the maintenance of reproductive stems per plant (tillers) was an important determinant of salt tolerance (Katerji et al., 2005).

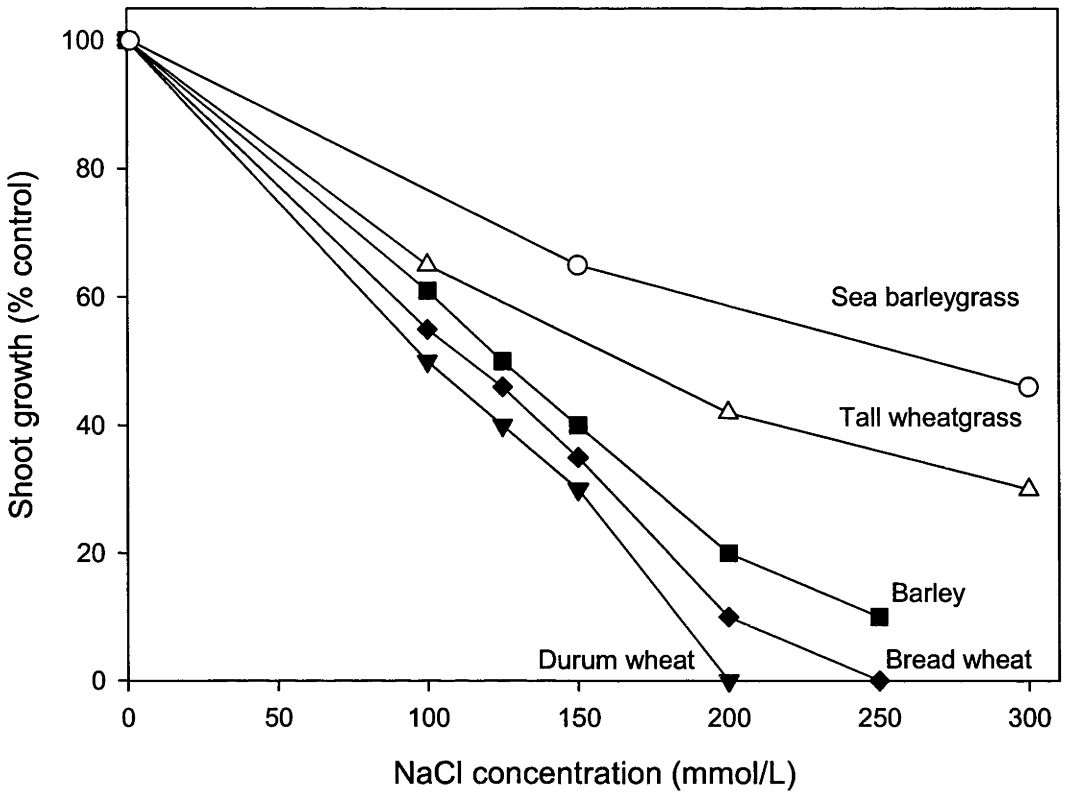

Figure 1.3 Salt tolerance of durum wheat compared to bread wheat, barley, Tall wheatgrass and Sea barleygrass. Reproduced from Colmer et al. (2005)

Na^+ and K^+/Na^+ selectivity in salt-stressed wheat

Durum wheat, a tetraploid species (28 chromosomes, 2 genomes: AABB), typically has higher rates of Na^+ accumulation than bread wheat, which is a hexaploid species (42 chromosomes, 3 genomes: AABBDD). The lower salt tolerance of durum wheat is considered to be mainly due to these high rates of Na^+ accumulation in the leaves and poor K^+/Na^+ discrimination (Gorham et al., 1987; Gorham et al., 1990b; Ashraf and O'Leary, 1996). For example, in a study comparing a bread wheat (Kharchia) with a durum wheat (HD45020), Joshi et al. (1982) found a large reduction of 56% in yield of salt-stressed durum wheat was associated with a 6 fold higher Na^+ concentration and Na/K ratio compared to the bread wheat with a yield reduction of only 11%. Similarly, variation in Na/K ratios was related to variation in salt tolerance among six bread wheat varieties Chippa and Lal (1995).

A locus for Na^+ exclusion and enhanced K^+/Na^+ discrimination in hexaploid wheat, called *Kna1*, has been mapped to the long arm of chromosome 4D (Dubcovsky et al., 1996), the genome lacking in tetraploid wheat. Recently, a novel tetraploid landrace (Line 149) has been identified with low Na^+ accumulation and high K^+/Na^+ discrimination. This trait has the potential for improving salt tolerance of durum wheat cultivars through conventional breeding (Munns et al., 2000b). Genetic analysis of a cross between the low Na^+ Line 149 and a high Na^+ durum cultivar Tamaroi, indicated two genes of major effect for Na^+ exclusion (Munns et al., 2003). A comparative glasshouse growth study at moderate salinities found a 20% yield advantage associated with Line 149 compared to a high Na^+ durum landrace (Husain et al., 2003), indicating the potential for increasing yields of durum wheats in moderately saline soils.

While there is often a correlation between low Na^+ and high K^+/Na^+ ratios and salt tolerance in wheat, this was not always he case with a number of studies indicating that other factors may be responsible for variation in salt tolerance (Ashraf and McNeilly, 1988; El-Hendawy et al., 2005b). Recently, Royo and Abió (2003) have shown this lack of association between shoot ion contents and yield of 17 salt-affected durum genotypes. This study showed there is potential for increasing the salt tolerance of durum wheat through traits other than Na^+ exclusion, such as Na^+ tissue tolerance of high salt concentrations as found in barley.

Use of progenitors and wild relatives of wheat to improve salt tolerance

The potential of progenitors and wild relatives to improve the salt tolerance of wheat has been review recently (Colmer et al., 2005; 2006). Figure 1.3 shows the greater degree of salt tolerance of two wild halophytic relatives of wheat and barley, Tall wheatgrass (*Thinopyrum ponticum*) and Sea barleygrass (*Hordeum marinum*). There may be additional variation in wheat progenitors and related wild species for salt tolerance traits other than Na^+ exclusion and K^+/Na^+ selectivity that can be used to improve the salt tolerance of modern durum wheats. For example, in the diploid wheat *Aegilops tauschii*, a progenitor of the D genome in bread wheat, salt tolerance of some individuals did not correlate with leaf Na^+ concentrations, possibly indicating variation in the degree of cellular or tissue tolerance to Na^+. (Schachtman et al., 1991).

1.2 LITERATURE REVIEW

1.2.1 Factors that affect growth of plants in saline soil

The growth of crop plants in response to salinity can be described by a two-phase model (Munns, 1993). Growth is initially reduced, by the decrease in soil water potential (Figure 1.4). This is essentially a water stress resulting from the osmotic affect of salts in the soil solution, and is similar to that imposed by soil drying. Growth can also decrease as a result of the salts taken up by the plant and accumulating in the older leaves to toxic concentrations. This is the 2nd phase of the two-phase model and is termed the 'salt-specific effect' (Figure 1.4).

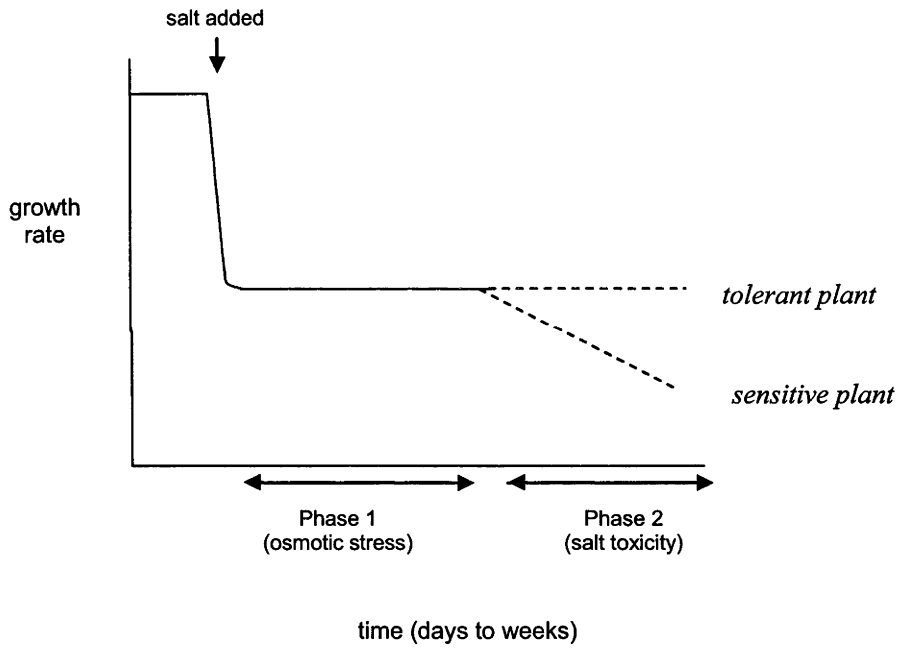

Figure 1.4 Schematic of two phase growth model in response to salinity (adapted from Munns, 1993)

Evidence supporting the two phase growth response model has also been shown in maize (Cramer et al., 1994a; Fortmeier and Schubert, 1995) and in wheat (Munns et al., 1995).

1.2.1.1 Osmotic stress

Initial salinity-induced reductions in leaf growth and stomatal conductance are due to factors associated with osmotic stress (Munns, 1993; Munns, 2002). The addition of

NaCl to the root medium causes an immediate decrease in leaf elongation rate (Termaat et al., 1985, Yeo at al. 1991; Fricke 2004; Fricke et al., 2004). This instant response (in minutes) is mediated through transitory changes in turgor, which can be overcome by placing the roots in a pressure chamber and maintaining the leaf xylem sap at atmospheric pressure (Passioura and Munns, 2000). However, over the timescale of days, this method of maintaining the turgor of the shoot failed to stop a reduced steady rate of leaf growth, leading to the hypothesis that hormonal signals, presumably from the roots were controlling growth and not a leaf water deficit (Termaat et al., 1985; Munns et al., 2000a). These early changes in growth are not related to increased Na^+ and Cl^- concentrations in growing tissues. There have been a number of studies in wheat (Hu and Schmidhalter, 1998), barley (Munns et al., 1988; Fricke et al., 2004) and maize (Neves-Piestun and Bernstein, 2005), showing that Na^+ and Cl^- concentrations in the rapidly dividing and expanding leaf tissues were well below toxic levels (~ 40 mM). Further, similar early decreases in growth have been found using osmotica other than NaCl (Delane et al., 1982; Termaat and Munns, 1986). However, in contrast to a majority of studies, Sümer et al. (2004) found early decreases in maize leaf growth associated with both osmotic and salt-specific (Na^+) effects.

It is likely that these same factors controlling growth of plants under osmotic stress are also regulating stomatal conductance. Over the timescale of days the reduction in growth rate is often matched by a reduction in stomatal conductance. For example, in spinach grown in 200 mM NaCl, stomatal conductance and growth were equally affected, with a decline of about 65%, relative to non-salt controls (Robinson et al., 1983). Similar results have been shown in salt-stressed wheat and barley (Termaat et al., 1985; Fricke et al., 2004; El-Hendawy et al., 2005b), rice (Yeo et al. 1985) and in bean (Montero et al., 1998; Sibole et al., 1998).

1.2.1.2 Salt toxicity

Over time in salinity (weeks to months), injury can be seen in older leaves followed be leaf senescence and death. If the rate of leaf death due to salinity in the mature older leaves is comparable to or exceeds the rate that new leaves are formed, there would be a substantial reduction in supply of assimilate to the growing regions, and therefore growth and potentially yield would be affected (Munns, 1993; Munns et al., 1995).

Cereals such as wheat and barley exclude at least 94% of the Na^+ in the soil solution (Section 1.2.2.2). The Na^+ that is not excluded by the roots accumulates

primarily in the older mature leaves which have been transpiring for longer periods of time. Once the capacity of the vacuoles in the leaves to compartmentalise salt ions is exceeded, salts will build up in the cytoplasm, resulting in the disruption of various enzymatic processes leading to the impairment of important physiological and biochemical processes (Munns, 1993). Alternatively, salts might build up in the cell walls and dehydrate the cell (Flowers and Yeo, 1986). The inevitable outcome of either of these processes is accelerated cell death, leaf injury and ultimately, leaf senescence.

1.2.2 Mechanisms for salt tolerance

Salt tolerance depends upon maintaining green leaf area, both in terms of rate of photosynthesis per unit leaf area and total photosynthesising area, thus maintaining an adequate supply of carbon to growing tissues and reproductive structures. For crop plants such as wheat and barley, this will result in the initiation and growth of tillers and the subsequent supply of assimilate to fill ears. Mechanisms for salt tolerance in (glycophytic) crop plants fall into three main categories: 1) tolerance to osmotic stress, 2) reducing salt entry and build up in leaves through salt exclusion processes and 3) the minimisation of salt build up in the cytoplasm through efficient vacuolar compartmentation, called tissue tolerance (Munns, 2002). Little is known about the mechanisms for tolerance to osmotic stress which is discussed first.

1.2.2.1 Tolerance to osmotic stress

Mechanisms for regulation of growth rate and stomatal conductance in salt and drought stressed plants are unclear.

Evidence for a hormonal root signal regulating transpiration rate has been found in drought-stressed wheat seedlings (Gollan et al., 1986; Passioura, 1988). The impact of hormonal control of cell division and differentiation is apparent from the appearance of salt-affected leaves which are smaller in area but often thicker, indicating that cell size and shape has changed (Munns, 2002). The stress hormone abscisic acid (ABA) is a likely candidate for the hormone which regulates growth under water stress conditions (Munns and Cramer, 1996). Further, the role of ABA in controlling stomatal conductance of plants grown in drying or saline soils is also well documented (Davies and Zhang, 1991). ABA was correlated with leaf growth inhibition in maize (Cramer et al., 1998; Cramer and Quarrie, 2002) and correlations between leaf and xylem ABA and both leaf expansion rate and stomatal conductance have been shown in salt-stressed

bean (Montero et al., 1998; Sibole et al., 1998). Whether ABA is the signal from the roots or is synthesised in the leaves in response to other unknown signals is still a matter of debate (reviewed by Dodd, 2005).

Genetic variation in tolerance to osmotic stress?

In proposing the two-phase growth response model to salinity, Munns (1993) also provided evidence from a number of studies that there was little genotypic variation in the growth due to the osmotic component of salt stress. As a consequence, genotypic differences in growth are likely to appear up later, be a result to leaf death and be associated with either Na^+ exclusion or vacuolar compartmentation capacity (Figure 1.4).

This first part of this model was challenged by Neumann (1997) who cited a number of cases to the contrary in salt-stressed maize (Cramer et al., 1994a; Mladenova, 1990) wheat (Blum et al., 1980; Kingsbury et al., 1984) and rice (Aslam et al., 1993; Moons et al., 1995). However, there is a degree of uncertainty with some of these examples, due to the growth conditions, especially high temperatures which would increase ion uptake (Munns et al., 1995), differences in the duration of these studies and also whether the differences in growth between genotypes was due to more efficient cellular compartmentation. Additionally, the onset of salt-specific phase in salt-sensitive species such as rice may be earlier than 'weeks to months' due to higher rates of Na^+ uptake and accumulation in leaves due to transpirational by-pass flow.

Some recent studies have also indicated the possibility of genetic variation in osmotic stress tolerance in wheat (El-Hendawy et al., 2005b) and barley (Jiang et al., 2006b), but again, are difficult to evaluate because of confounding factors. For example, El-Hendawy et al. (2005b) revealed significant genotypic variation in stomatal conductance and relative growth rate (RGR) amongst 13 wheat varieties under a range of salt treatments. Reductions in stomatal conductance at 150 mM NaCl were in the order of 35% for salt-tolerant varieties and 60% for sensitive varieties, relative to controls. However, the concurrent increase in chlorophyll in some varieties with increasing salinity indicated a decrease in leaf area and thereby possibly a higher density of stomata per unit leaf area. This could explain the higher conductance values as long as the higher density was not balanced by a reduction in stomatal size or aperture.

That said, it is apparent that genotypic variation in salt tolerance associated with tolerance to osmotic stress is an area that is unclear and largely untested. Care needs to be taken in designing experiments and interpreting results on the contribution of osmotic stress to variation in growth and stomatal conductance of salt-stressed plants.

1.2.2.2 Salt exclusion

Genotypic variation in salt tolerance within most cereal species has been characterised on the basis of Na^+ exclusion capability. All crop plants exclude at least 94% of Na^+, with bread wheats excluding up to 99% while durum wheat and barley do not exclude as well with 94 - 96% exclusion (summarised in Munns, 2005). The degree of salt tolerance has been found to correlate inversely with Na^+ accumulation in the leaves of many species including barley (Greenway, 1962), rice (Flowers and Yeo, 1981), diploid wheat (Schachtman et al., 1991) and bread wheat (Munns and James, 2003). Salt tolerance in a crop species such as wheat is usually associated with reduced uptake of Na^+ and maintenance of high K^+ in the shoots (Gorham et al., 1990b); i.e. the maintenance of high K^+ concentrations and the resulting high K:Na ratio in the leaves is as important as reduced Na^+ uptake into the leaves (see sections below).

In the Triticeae, salt toxicity is considered to be associated with Na^+ rather than Cl^-. In contrast to Na^+, there is little genetic variation for Cl^- accumulation in wheat (e.g. Gorham, 1990). Also, Cl^- is largely partitioned to epidermal cells and therefore does not build up to toxic levels in the more metabolically important mesophyll cells (Huang and van Steveninck, 1989; Leigh and Storey, 1993; Fricke et al., 1996).

Mechanisms which regulate Na^+ uptake, transport and eventual deposition into leaves have been extensively reviewed over the last 25 years (e.g. Greenway and Munns, 1980; Läuchli, 1984; Cheeseman, 1988; Amtmann and Sanders, 1999; Hasegawa et al., 2000; Tester and Davenport, 2003; Munns, 2005). There are three main mechanisms which control or regulate net uptake of Na^+ into the leaves of crop species:

1. Control of net Na^+ uptake and transport in the roots
2. Partitioning of Na^+ in the shoot
3. Retranslocation of Na^+ from the shoot to the roots

Control of net Na^+ uptake and transport in the roots

The roots are the primary site for the regulation of Na^+ accumulation in leaf blades and there are a number of control points in the roots which collectively regulate the concentration of Na^+ in the transpiration stream. Control of net Na^+ uptake into the roots is largely a function of rates of unidirectional influx and efflux of Na^+. Unidirectional influx rates are high and likely to be the result of non-selective cation channels (Demidchik et al., 2002). Efflux rates out of the roots through the cortical cells are also high and are thought to be mediated by Na^+/H^+ antiporters (Blumwald et al., 2000; Tester and Davenport, 2003). Recently, Davenport et al. (2005) found little difference in efflux rate between two genotypes that differed in net Na^+ transport into the shoot. This indicates that Na^+ transport from the root to the shoot is therefore largely a function of the control of loading of Na^+ into the xylem and its subsequent retrieval from the xylem. Mechanisms which control Na^+ loading into the xylem are currently unknown. Na^+ could move out of the xylem passively, via a Na^+ permeable channel or a Na^+ uniporter into the xylem parenchyma, but only if Na^+ is high in the xylem and low in the cytoplasm. Candidate transporters for passive withdrawal of Na^+ from the xylem include non-selective cation channels and high affinity K^+ transporters (HKTs) that function as Na^+ uniporters in a low affinity mode (Tester and Davenport, 2003; Rodríguez-Navarro and Rubio, 2006).

Partitioning of Na^+ in the shoot

Growing and photosynthetically active leaf tissue and reproductive structures could be protected from the effects of high salt concentrations through the preferential partitioning of salts into physiologically less metabolically active shoot tissues such as the stem, the leaf sheath and older leaves. There are a number of studies showing the preferential partitioning of salt ions into the sheaths and stems or the basal part of the shoot. For example, Na^+ in the leaf base of rice and the reed plant *Phragmites communis* (Matsushita and Matoh, 1991) and sorghum (Lacerda et al., 2003) and Cl^- into the leaf sheaths has been identified in wheat, maize sorghum and barley (Boursier et al., 1987; Huang and van Steveninck, 1989). Recently, a novel low-Na^+ durum wheat (Line 149) was identified which was able to remove Na^+ from the xylem into the leaf sheath (Davenport et al., 2005). Additionally, the Na^+ levels in the leaves of Line 149 grown in 150 mM NaCl became progressively smaller in successive leaves up the main stem, possibly indicating a greater involvement of the sheath ('stem') in sequestering and

storing Na^+ from the xylem stream (Munns et al., 2003; Husain et al., 2003). Similarly, Wolf et al. (1991) found decreasing Na^+ concentrations in the xylem sap between the basal part of the elongating stem and upper part.

Retranslocation of Na^+ from the shoot to the roots

The retranslocation of Na^+ from the shoot to the roots may also contribute to the maintenance of low Na^+ concentrations in the leaf blade. However, the relative contribution of this mechanism in crop plants such as barley and wheat is usually considered to be small. Several studies have shown that phloem export from the shoot in barley is no more than 10 – 15% of the transport of Na^+ into the shoot (Munns et al., 1986; Wolf et al., 1990)) and similar values (between 5 – 10%) were estimated in durum wheat using a split root system fed $^{22}Na^+$ (James et al., 2006). Lower leaves and basal stem tissues are the main source for Na^+ movement from the shoot to the roots. Retranslocation of Na^+ that was deposited in the shoot base was shown in *Phaseolus vulgare* (Jacoby, 1979) and the reed plant *Phragmites communis* (Matsushita and Matoh, 1991) and from the lower leaves and sheaths of barley and durum wheat (Wolf et al., 1991; James et al., 2006).

1.2.2.3 Tissue tolerance

A number of studies have found that Na^+ concentrations in the leaves did not always predict the salt tolerance. While Yeo and Flowers (1983) found an inverse relationship between Na^+ content and chlorophyll in leaf 3 in rice, they also found 3 fold genotypic variation in the amount of Na^+ in the leaf that corresponded to a 50% loss in chlorophyll. These authors suggested that there was variation amongst varieties in the 'resistance' to Na^+ building up in the leaf tissue. Similarly, in diploid wheat (Schachtman et al., 1991) and maize (Cramer et al., 1994b), salt tolerance of some individuals did not correlate with leaf Na^+ concentrations, possibly indicating variation in the degree of cellular or tissue tolerance to Na^+.

Tissue tolerance depends largely on the capacity to compartmentalise salt ions into safe storage places such as vacuoles. This mechanism therefore prevents the breakdown of key physiological processes such as photosynthesis through membrane damage or enzyme inhibition when salts may build up to toxic levels in the cytoplasm (Greenway and Munns, 1980; Munns, 1993).

Tissue tolerance of high Na^+ concentrations is likely to be a complex trait, one that is difficult to quantify and which may be confounded by interactions with

environmental conditions, phenology and plant vigour. For example, Yeo et al. (1990) used the relationship between chlorophyll degradation and leaf Na^+ concentration as a quantitative measure of tissue tolerance in rice. While these authors found a five fold range in tissue tolerance amongst 21 rice accessions, they could find no clear correlation with key performance indicators such as survival or vigour, and also concluded that other characteristics such as plant height confounded the study.

Barley – a tissue tolerant ideotype

Barley is usually considered as one of the more salt-tolerant crops, more tolerant than hexaploid and tetraploid wheat (Maas and Hoffman, 1977; Rawson et al., 1988b). However, little is known about the mechanisms that confer salt tolerance in barley, since it accumulates high levels of tissue Na^+. There is some evidence to suggest that the higher tolerance of grain yield in barley is a result of accelerated leaf area development and shorter flowering time (Rawson et al., 1988b; Richards et al., 1987). These features may also be advantageous in increasing water use efficiency and therefore have the potential to increase the productivity of barley grown in saline soils (Richards, 1992). Nevertheless, while Rawson et al. (1988b) found genotypic differences in biomass production in salinity between wheat, barley and triticale cultivars, the barley cultivars were in the main, still more salt-tolerant than the bread wheat cultivars even when developmental differences were accounted for.

Salt tolerance in barley is not usually linked to the ability to exclude salts from the shoots. While some genotypic variation in leaf Na^+ (Forster et al., 1994) and Cl^- (Greenway, 1962) exists, barley generally has high Na^+ and Cl- concentrations in the leaves compared to hexaploid wheat (Gorham et al., 1990a; Forster et al., 1994; Rawson et al., 1988b). Additionally, Royo and Aragüés (1999) found poor and inconsistent correlations between Cl^-, Ca^{2+}, Na^+ and K^+ in leaf sap and performance indicators such as yield, in a study using about 50 barley varieties. The enhanced K/Na discrimination trait also appears to be absent in barley and wild barley ancestors (e.g. *H. spontaneum*) compared to bread wheats; however, in contrast, a recent study on 70 barley cultivars concluded that maintaining a high K/Na ratio in leaves was a key feature of salt tolerance (Chen et al., 2007). Gorham et al. (1990a) found high Na^+ and low K^+ concentrations and a correspondingly low K:Na ratio of 0.85 in 13 barley accessions grown in 160 mM NaCl. This was similar to the K:Na ratio of durum wheats tested (0.57), but contrasted significantly to the bread wheats (~ 6.0). These authors suggested

that salt tolerance in barley cultivars may be due to the ability to partition Na^+ within the shoot to the older leaves and K^+ to the younger growing leaves and also to efficiently compartmentalise Na^+ and K^+ (and compatible organic solutes) within the leaf. These characteristics have not yet been firmly established in barley, and nor have factors controlling tissue tolerance in tetraploid or hexaploid wheat.

Factors that may affect tissue tolerance

If crop species such as barley and durum wheat are grown in saline soil, Na^+ arrives at the leaves via the transpiration stream in relatively high concentrations (2-3 mM) due to an inability by the roots to restrict the net influx into the xylem. This is in contrast to bread wheat, where a more efficient control of Na^+ entry into (or removal from) the xylem is responsible for keeping xylem Na^+ concentrations less than 1 mM (Gorham et al., 1990b; Munns, 2005). There are a number of processes which may allow the leaves of barley and (perhaps) durum wheat to cope with the subsequent build up of Na^+ to high concentrations. These are; cellular ion compartmentation, compatible solutes and osmotic adjustment, and the relevance of Na^+ and Cl^- in the apoplast.

Cellular ion compartmentation

Tolerance to high internal Na^+ concentrations in leaves depends on coordinated cellular and subcellular partitioning to efficiently sequester Na^+ and Cl^- into cell types such as the epidermis and cellular compartments such as the vacuole, thus minimising accumulation in the cytoplasm. Similarly, partitioning of K^+ is also required to maintain adequate K^+ in the cytoplasm.

Patterns of cellular ion accumulation in the leaves of salt-affected barley have been examined in a number of studies, using a variety of analytical techniques including X-ray microanalysis (e.g. Huang and van Steveninck, 1989; Leigh and Storey, 1993), vacuolar sap extraction (e.g. Fricke et al., 1996), and isolated protoplasts (e.g. Dietz et al., 1992). Distinct preferential partitioning of Cl^- to epidermal cells was evident in all these studies. This pattern of preferential epidermal Cl^- deposition has also been established in non-saline conditions (Huang and van Steveninck, 1989; Fricke et al., 1996; Karley et al., 2000a).

A consistent pattern for the cellular distribution of Na^+ has not been so apparent. Contrasting partitioning patterns were revealed by different studies using different techniques and for different salinity levels within the same study. Using a

microcapillary to extract sap from single cells, Fricke et al. (1996) found higher Na^+ concentrations in epidermal vacuoles than mesophyll vacuoles in barley grown in 50 – 150 mM NaCl. In contrast, Huang and van Steveninck (1989) found that the mesophyll of barley contained twice the Na^+ concentration to that of the epidermis, after 4 d in 50 mM NaCl. Similarly, Leigh and Storey (1993) encountered more mesophyll than epidermal cells with detectable levels of Na^+. Potassium appears to accumulate similarly in both mesophyll and epidermal cells under non-saline conditions or at low salinities and generally appears to accumulate preferentially in mesophyll cells under high salinities (Leigh and Storey, 1993; Fricke et al., 1996; Cuin et al., 2003).

At what concentration does Na^+ become toxic?

Tolerance to high internal salt concentrations in the leaves is thought to be linked with low Na^+ and high K^+ concentrations in the cytoplasm (Leigh and Wyn Jones, 1984; Munns, 1993; Maathuis and Amtmann, 1999). The toxicity of high Na^+ concentrations in the cytoplasm mostly relates to its ability to compete with K^+ for catalytic sites. As a result, high Na^+:K^+ ratios will lead to the disruption of many important K^+ dependent enzymatic processes, which ultimately affect growth (Munns et al., 1983; Maathuis and Amtmann, 1999; Tester and Davenport, 2003).

It is not clear what actually constitutes a toxic concentration for Na^+ (Cheeseman, 1988). This may be related in part, to the difficulty of accurately measuring ion concentrations in the cytoplasm, which is a small and narrow cellular compartment. Munns (1993) suggests that non-toxic cytoplasmic concentrations of Na^+ in leaves could possibly be as high as 100-150 mM. In contrast, Tester and Davenport (2003) concluded that cytoplasmic Na^+ concentrations could be as low as 10 to 30 mM in the roots of plants grown in external salinities as high as 200 mM NaCl.

Critical information that is still lacking is the relationship between key physiological processes, such as photosynthesis, and cellular ion compartmentation and the threshold at which Na^+ and Cl^- become toxic in the cytoplasm in salt-stressed plants. Few studies have attempted to link these parameters. In a study examining the relationship between photosynthesis and ion content in salt-stressed spinach, Robinson et al. (1983) suggested that Na^+ concentrations of 345 mM in the leaf and 165 mM in isolated chloroplasts did not result in any major decrease in the photosynthetic potential.

Fricke et al. (1996) concluded that large increases in vacuolar mesophyll Na^+ (~ 300 mM) and Cl^- (120 -170 mM) concentration were not associated with a relatively small

reduction in photosynthesis (17%) of barley grown at 150 mM NaCl. However, in that study, very little information was given relating to the details of the photosynthesis measurements. It seems likely that the gas exchange measurements were completed on leaves that developed in the presence of the salt stress. Photosynthetic capacity may have increased in these leaves if the salt stress reduced leaf size without reducing the amount of N per leaf. This would mask the full effects of the salt stress. Another study by Seemann and Critchley (1985) on salt-stressed *Phaseolus,* found similar Cl^- concentrations (250-300 mM) in both the cytoplasm (and chloroplasts) and in the vacuole indicating a breakdown in vacuolar compartmentation. These authors suggested that these high Cl^- concentrations affected either the efficiency or the activity of RuBP carboxylase by as much as 40%.

Relationship between ion compartmentation and salt tolerance

There are few studies examining the relationship between cellular or subcellular ion compartmentation and salt tolerance which have utilised the comparison of varieties differing in salt tolerance. One comparative study by Huang and van Steveninck (1989) assessed the differences in the Na^+ and Cl^- vacuolar concentrations in mesophyll and epidermal cells of a salt-tolerant barley cv. California Mariout and salt-sensitive barley cv. Clipper (see Rawson et al., 1988b) using X-ray microanalysis. These authors concluded that the lower salt tolerance of Clipper was related to higher Cl^- concentrations in the vacuoles of mesophyll cells compared to California Mariout. A closer examination of this work reveals that while there were significant genotype differences in vacuolar mesophyll Cl^- concentration, both genotypes were at very low and non-toxic concentrations, 4 - 8 mM (California Mariout) compared to 27 – 44 mM (Clipper).

The only other study of this kind was completed on two other barley genotypes, Gerbel and Triumph, which differed in salt tolerance (Flowers and Hajibagheri, 2001). Using X-ray microanalysis to measure ion concentrations in root cortical cells, the salt-tolerant barley variety Gerbel appeared to be more effective at excluding Na^+ from the cytosol and sequestering it in the vacuoles compared to the salt-sensitive variety Triumph. Presumably due to large variation between plants and in sampling the various compartments, the 30 – 35% errors associated with these measurements meant that while these observations were interesting they were not statistically significant. However, these conclusions were confirmed on these same varieties in a later study

(albeit also with large errors) using K^+ and Na^+-selective microelectrodes (Carden et al., 2003). These authors found that Gerbel was also better at maintaining cytosolic a_K which resulted in a 10 fold increase in the cytosolic K^+:Na^+ over Triumph after 5 d at high salinity.

How much these studies on roots can tell us about the mechanisms of cellular and subcellular compartmentation in the leaf is uncertain. It does seem certain that higher cytosolic Na^+ concentrations in the root will result in higher Na^+ uptake into the leaves, but how much of this Na^+ ultimately builds up in the cytoplasm of mesophyll cells will depend on a number of ion transport processes controlling both ion supply and uptake into specific cell types (Karley et al., 2000b).

These studies also highlight the difficulties in obtaining accurate measurements of ion concentrations in cell compartments, particularly the cytoplasm. The techniques described that have been used all have limitations (e.g. the number of cells that can be realistically sampled) and their characteristic sources of error (e.g. artefacts derived from sample preparation and measurement).

Compatible solutes and osmotic adjustment

If Na^+ and Cl^- are preferentially accumulated in the vacuole, K^+ and organic solutes must increase in the cytoplasm and organelles to balance the osmotic pressure of the ions in the vacuole. The balancing organic solutes are commonly called compatible solutes or osmolytes, and can be classified under four classes; 1) betaines and structural analogues (e.g. glycinebetaine), 2) polyhydric alcohols (e.g. mannitol), 3) sugars (e.g. sucrose), and 4) amino acids (e.g. proline) (Colmer et al., 2005). In the Poaceae the compatible solutes that accumulate most commonly under abiotic stress are glycinebetaine and proline (Storey et al., 1977; Rhodes and Hanson, 1993). Proline appears to increase predominantly in response to water stress (drought), whereas glycinebetaine increases in response to salt stress (Wyn Jones and Storey, 1978a; Sabry et al., 1995).

At the whole plant level, glycinebetaine accumulates primarily in the younger leaves of salt-stressed wheat, barley and sorghum plants (Colmer et al., 1995; Nakamura et al., 1996; Yang et al., 2003), whereas proline builds up in older leaves (Colmer et al., 1995). At the cellular level, compatible solutes accumulate primarily in the cytosol, including organelles, and are largely absent from the vacuole (Leigh et al., 1981; Matoh et al., 1987). Chloroplasts in particular appear to contain a large proportion of the

glycinebetaine found in a leaf, and due to the small size of these organelles, concentrations are calculated to be very high (e.g. ~ 300 mM in salt-stressed spinach – Robinson and Jones, 1986). The osmoprotective ability of compatible solutes is largely speculative. Apart from contributing to the positive water status and therefore the integrity of the cytoplasm and organelles, compatible solutes are also thought to protect enzymes (e.g. Rubisco) and membranes from high ion concentrations and have cryoprotectant and heat-protectant properties (summarized by Gorham, 1995).

There is evidence for genotypic variation in glycinebetaine levels in barley, maize and sorghum. While only a 2 - 3 fold range in gylcinebetaine levels was found in non-stressed barley cultivars (Grumet et al., 1985; Grumet and Hanson, 1986), a 200 fold range was found in a large collection of 240 sorghum genotypes screened under non-stressed conditions (Yang et al., 2003). Similar screening of large wheat collections has not been undertaken, but it is likely that useful variation could also exist (Sabry et al., 1995), which may provide the potential to increase salt tolerance of current wheat varieties. For example, Colmer et al. (1995) concluded that accumulation of glycinebetaine (together with the maintenance of low Na^+ and high K^+ contributed to the salt tolerance of a wheat (cv. Chinese Spring) x *Lophopyrum elongatum* amphiploid. Similarly, the pre-treatment of wheat seedlings with glycinebetaine (but not proline) fully alleviated salt-induced limitations on photosynthesis (Rajasekaran et al., 1997). Interestingly, Szegletes et al. (2000) found higher levels of betaine and proline in drought stressed Indian wheat variety Kharchia – well known for salt tolerance – compared to a small collection of drought tolerant and sensitive wheats.

Relevance of salt ions in the apoplast

Tolerance to high internal salt concentrations in the leaves is also thought to be linked with the maintenance of low Na^+ concentrations in the apoplast (Oertli, 1968; Munns and Passioura, 1984; Mühling and Läuchli, 2002a). As solutes leave the xylem, they are likely to move through the mesophyll in the symplast, and not in the apoplast (Canny, 1990). The apoplastic Na^+ concentration will depend on the cytoplasmic concentration, which in turn is related to both the duration of salt uptake by the leaf and the storage capacity of the vacuoles. Once the vacuolar storage capacity of mesophyll cells is reached at high leaf Na^+ concentrations, excess salt ions may be effluxed out of the cell and quickly build up to high concentrations in the cells walls, leading to turgor loss and cell dehydration (Munns, 1993).

Apoplastic Na^+ concentrations are usually assumed to be low. For example, apoplastic Na^+ remained below 10 mM in both salt-stressed wheat (Wimmer et al., 2003) and barley (Ramanjulu et al., 1999) grown in 75 mM and 100 mM NaCl respectively. Additionally, differences in apoplastic Na^+ do not appear to be reliably predictive of salt tolerance. Two inter-specific studies examining the relationship between salt tolerance and differences in apoplastic Na^+ concentrations found conflicting results. Using the infiltration-centrifugation method, Speer and Kaiser (1991) found higher apoplastic Na^+ concentrations in the salt-sensitive pea (87 mM) than in the more salt-tolerant spinach (7 mM) grown in 100 mM NaCl . In contrast, Mühling and Läuchli (2002a), also using the infiltration-centrifugation method, found higher apoplastic concentrations in the older leaves of salt-tolerant cotton (12mM) than in the salt-sensitive corn (~3 mM) grown in 75 mM NaCl for 14 d. Further, no significant differences were found in apoplastic Na^+ concentrations in cotton grown at 75 and 150 mM NaCl, while yield declined at the higher salt treatment (Mühling and Läuchli, 2002b). Collectively, these findings suggest that the reported Na^+ concentrations may not have been high enough in the apoplast to cause dehydration of leaf cells which would lead to inevitable cell death and leaf senescence. Only Speer and Kaiser (1994) have attempted to link visible symptoms of leaf injury with apoplastic Na^+ concentrations. These authors concluded that apoplastic ion accumulation was not the cause, but rather a consequence of salt damage to the leaf.

1.3 RATIONALE AND AIMS

1.3.1 Rationale

Large areas in the Australian wheat belt are characterised with subsoil or seepage salinity which profoundly affects the development and subsequent yield of modern durum cultivars. The development of salt-tolerant durum lines would provide growers with an opportunity to successfully grow a premium wheat grade over wider salt-affected areas.

Durum and other tetraploid wheats are typically very salt sensitive compared to hexaploid bread wheat. This is largely due to poor sodium exclusion, a character thought to be confined to the D genome, and therefore not present in durum wheat. Recent research has found that there is genetic variation for sodium exclusion in tetraploid wheat and progress has been made to find molecular markers and incorporate the genes responsible into current cultivars and breeding lines. This could make durum wheat as tolerant to salinity as bread wheat. However, further improvements are also possible.

Tolerance to high internal concentrations of sodium in the leaf is recognized as another important mechanism for salt tolerance. This trait is yet to be identified in tetraploid wheat; however, it is present in barley and genetic variation has been found to exist in rice and in the diploid D genome wheat progenitor. Tissue tolerance cannot be measured directly, is difficult to quantify and yet it is clearly an important component of salt tolerance. It is envisaged that improvements in sodium tissue tolerance may produce enhanced salt tolerance in durum wheat, particularly in soils with high salinity where Na^+ exclusion may prove ineffective.

Tolerance to the osmotic stress associated with salinity also has the potential to provide an avenue to improve growth and yield of durum wheat grown in saline soils. Osmotic stress from salinity affects the growth all crop plants, irrespective of any ability to exclude salt accumulation in the shoot. Genetic variation for tolerance to osmotic stress in hexaploid wheat is assumed to be small, although there is some evidence in the literature to suggest otherwise. The presence of this trait has not been explored in tetraploid wheat.

The development of screening protocols and introduction of salt-tolerance traits into modern durum wheats is contingent on the fact that diversity exists within

tetraploid wheat for these salt tolerance traits. The development of screening techniques for salt tolerance traits based on a sound physiological framework will be critical for the effective identification of genetic diversity, and incorporation of these traits in modern durum cultivars.

1.3.2 Aims

The aims of this thesis fall into four broad categories:

1) Characterise genetic variation in tetraploid wheat in ability to tolerate high internal sodium.

2) Define physiological and biochemical mechanisms of tolerance to high internal levels of sodium and evaluate impact on plant performance.

3) Characterise genetic variation in tetraploid wheat in ability to tolerate the osmotic stress associated with salinity and evaluate impact on plant growth

4) Examine the potential to develop quick and reliable screening protocols that can accelerate the introduction of these traits into current durum cultivars and breeding lines.

1.3.3 Experimental framework of the thesis

The experiments described in Chapter 2 assess tissue tolerance to high internal salt concentrations in a diverse collection of tetraploid wheats (cultivars and landraces). These experiments identified five tetraploid landraces that maintained a large proportion of green leaf area despite high leaf Na^+ concentrations.

In the second part of Chapter 2 and in the following two chapters, the physiology of tissue tolerance and potential mechanisms involved in tissue tolerance in durum wheat are examined in some detail. The possible contribution of compatible solutes, glycinebetaine and proline to osmotic adjustment and tissue tolerance is assessed (Chapter 2).

A tissue tolerant line and a sensitive line that were identified are then used to examine physiological parameters including leaf ion accumulation, water relations, chlorophyll degradation, chlorophyll fluorescence and gas exchange (Chapter 3). This study examines the relative impact of stomatal and non-stomatal limitations on CO_2 assimilation of salt-stressed plants, and in particular the relationship between leaf ion

concentration and the onset of non-stomatal limitations as indicated by chlorophyll degradation and chlorophyll fluorescence parameters.

As a major component of tissue tolerance may be associated with the capacity to compartmentalise salt into vacuoles, the relationship between photosynthetic capacity and the cellular and subcellular distributions of Na^+, K^+ and Cl^- is compared in a tissue-sensitive durum wheat versus a tissue tolerant ideotype, barley (Chapter 4). Cryo-SEM X-ray microanalysis is used to examine vacuolar ion concentrations in the leaf and to calculate cytoplasmic ion concentrations. Leaf gas exchange measurements are used to estimate photosynthetic capacity and a decline in capacity parameters is related to ion concentrations in the cytoplasm.

The final series of experiments examines the expression of tolerance to (salt-induced) osmotic stress in durum wheat (Chapter 5). The response of stomatal conductance to salt stress, before salts build up in the leaf, is used to screen a large collection of international durum varieties. These screening experiments identified large genotypic variation in the magnitude of stomatal response to osmotic stress. This genotypic variation was then used to examine the relationships between stomatal conductance and relative growth rate and CO_2 assimilation rate.

In the final discussion, the significance of these results is discussed in the context of a growth response model to salinity, screening and breeding strategies for salt tolerance, and candidate genes that may contribute to tolerance. Some ideas for future work are presented.

Chapter 2

GENOTYPIC VARIATION IN TISSUE TOLERANCE TO NA^+ IN TETRAPLOID WHEAT

2. GENOTYPIC VARIATION IN TISSUE TOLERANCE TO NA^+ IN TETRAPLOID WHEAT

2.1 INTRODUCTION

One mechanism of salinity tolerance is tissue tolerance of high leaf Na^+ concentrations. This trait is exemplified by halophytes and is characterised by an absence of leaf injury despite high leaf concentrations of NaCl (Flowers et al., 1986). Concentrations of Na^+ above 100 mM will start to inhibit most enzymes *in vitro* (reviewed by Munns et al., 1983), so when tissue concentrations are over 100 mM, which corresponds to about 0.5 mmol g^{-1} DW (assuming a leaf water content of 5 g H_2O g^{-1} DW), there must be effective compartmentation of Na^+ in vacuoles. Halophytes have no metabolic adaptation to high salt, yet they can tolerate much higher Na^+ concentrations than 100 mM in the leaf (Greenway and Osmond, 1972; Flowers et al., 1986). Glycophytes have a degree of compartmentation ability, as levels of Na^+ up to 1 mmol g^{-1} DW are quite common in photosynthetically active leaves of many species. For example, in barley grown in 100 mM and 175 mM NaCl, leaf Na^+ concentrations between 200 – 400 mM (1 – 2 mmol g^{-1} DW) were not associated with reduced net photosynthesis rates (Rawson et al., 1988a). This indicates that barley must also have an ability to compartmentalise Na^+ into vacuoles, even at high leaf Na^+ concentrations.

When Na^+ and Cl^- accumulate in the vacuole, K^+ and compatible solutes such as sucrose, glycinebetaine and proline, must also increase in the cytoplasm and organelles to balance the osmotic pressure of the ions in the vacuole (Wyn Jones and Storey, 1978a). Leaf injury occurs when salts cannot be sequestered in vacuoles and therefore build up in the cytoplasm or cell walls (Munns 1993; Flowers and Yeo 1986).

Variation in salinity tolerance not associated with Na^+ exclusion was observed in accessions of the diploid wheat ancestor *Triticum tauschii* (syn. *Aegilops squarrosa*) (Schachtman et al., 1991), so it is possible that variation exists within the tetraploid wheat ancestors. To assess genetic variation in a representative set of selections from a range of tetraploid subspecies (relatives of durum wheat), leaf longevity, lack of necrosis, and prolonged growth despite very high accumulation of Na^+ were examined. However, a major limitation in the use of injury to identify salt tolerant germplasm arises when the cause of injury is not known. The injury could be due to water stress,

the Na^+ or Cl^- accumulating within the leaf, or nutrient imbalance (Greenway and Munns, 1980). Previous experiments at different root temperatures and light levels, showed leaf injury when high root temperatures were combined with high light and presumably high transpiration rate, but with little increase in Na^+ (Munns and James, 2003). This indicated that something other than Na^+ was causing leaf death. Elemental analysis of leaves showed that all elements except phosphorus (P) fell within recommended levels (Reuter and Robertson, 1986), indicating that P toxicity may contribute to leaf death in salt-treated plants. Therefore, the possible involvement of P toxicity to leaf injury of durum wheat grown in high salinity with a standard Hoagland's nutrient solution was assessed first, before screening for tissue tolerance. Lastly, the contribution of compatible solutes glycinebetaine and proline to salt tolerance was examined in a subset of putative tissue tolerant tetraploid lines.

2.2 MATERIALS AND METHODS

2.2.1 Germplasm

A collection of 47 *Triticum turgidum* selections comprising representatives from the sub-species *durum, turgidum, polonicum, turanicum* and *carthlicum* from the Australian Winter Cereals Collection, were provided by Dr Ray Hare (durum breeder, NSW DPI, Tamworth) as representing a wide range of genetic diversity (Table 2.1).

Table 2.1 Cultivars and numbers of tetraploid selections used in experimental series.

Genotypes	**Experiments**			
	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**
Selected tetraploid accessions:				
T. turgidum L. ssp. *durum* (Desf.)	3	1	17	2
T. turgidum L. ssp. *polonicum* (L.) Thell.	1	1	11	1
T. turgidum L. ssp. *turgidum*			7	1
T. turgidum L. ssp. *carthlicum* (Nevski)			6	1
T. turgidum L. ssp. *turanicum* (Jakubz.)			6	1
Durum wheat cultivars:	Wollaroi	Wollaroi	Wollaroi Tamaroi	Wollaroi Tamaroi
Bread wheat cultivars:	Janz	Janz	Janz Kharchia Westonia Machete	
Barley cultivar:			Skiff	Skiff
Total number of lines tested:	6	4	54	9

A complete list of these selections detailing name, sub-species and AUS number is listed in Appendix 2.1.

2.2.2 Growth conditions

Seeds were selected for uniform weight, surface sterilised with 1% hypochlorite for 15 min, and germinated on moistened filter paper in Petri dishes at room temperature for 3 d. Germinated seeds were planted 1.5 cm deep into 6.5 cm square x 15.8 cm deep pots containing quartz gravel, one plant per alternate pot, in 90 L plastic moulded trays containing 144 pots. Trays were subirrigated with either saline or non-saline nutrient solution, as described in Munns et al. (1995) and illustrated in Figure 2.1. This gravel culture was preferred to other forms of hydroponic culture, as each plant was a separate replicate, the pots were of adequate size, and there was no breakage of lateral roots as occurs in unsupported hydroponics when the solution is changed (Miller, 1987).

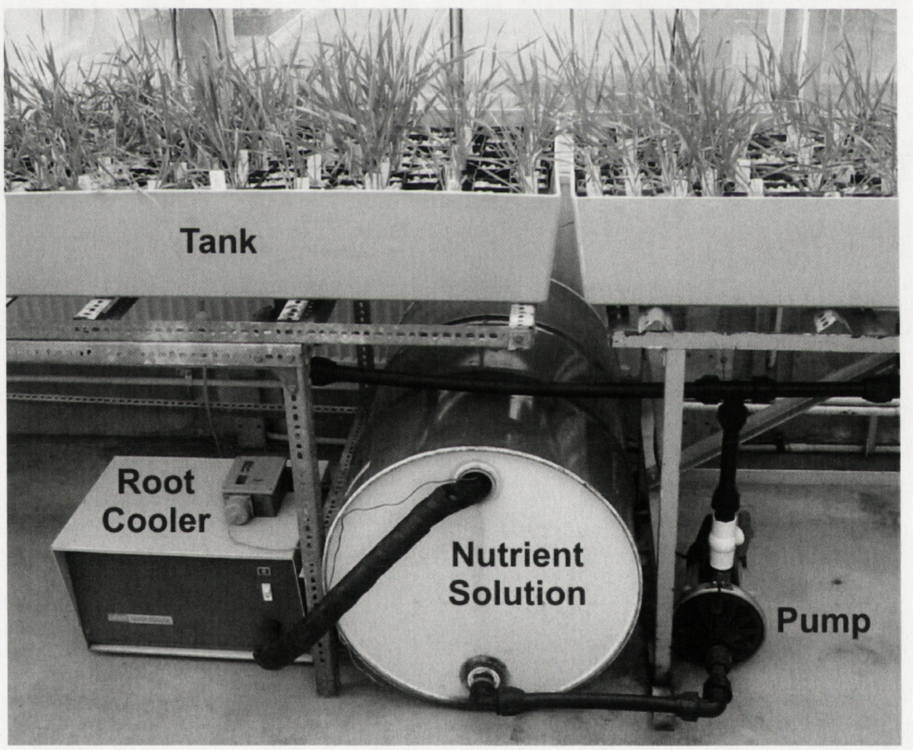

Figure 2.1 Supported hydroponic method for screening plants for salt tolerance. Pots are filled with quartz gravel and the tanks are subirrigated every 30 min with nutrient or saline solution from a reservoir, by a pump activated by a timer. A condenser unit was used to maintain solution temperature at about 20°C.

Seedlings were watered initially with half strength Hoagland's nutrient solution 2 days after emergence (DAE), which was increased to full strength Hoagland's solution (FSH) at 3 DAE. Commencing at 4-6 DAE, 25mM NaCl was added to the irrigation solution

twice daily over three days to achieve a final concentration of 150 mM. Supplemental Ca^{2+} was added (as $CaCl_2$) to bring the total concentration of Ca^{2+} to 10 mM, and the molar ratio of Na^+:Ca^{2+} to 15:1. Solution pH was measured twice weekly and adjusted as needed to pH 6.0 with 2 M HCl. Temperature of the solution around the roots was monitored every 5 min using thermocouples and controlled using condensers in the solution reservoirs. All experiments were conducted in a glasshouse with natural light and controlled air temperature (conditions during separate experiments are given below).

2.2.3 Effect of salinity and phosphorus on leaf injury (Exps. 1 and 2)

A selection of four *T. turgidum* lines, with bread wheat cultivar Janz and current Australian durum wheat cultivar Wollaroi (Table 2.1) were grown in two treatments consisting of 1) a control of 1 mM NaCl in full strength Hoagland's solution (containing 1 mM P) and 2) a salt treatment of 150 mM NaCl with 10 mM $CaCl_2$ in full strength Hoagland's solution (also containing 1 mM P)(Exp 1). Ten replicate seedlings per line were used for each treatment and replicates were randomly spaced within each treatment. Seedlings were harvested 22–24 DAE (corresponding to 15-17 d in treatment) and visually assessed for leaf injury (estimate of dead leaf as a percentage of total). Blades of leaf 1, 2 and 3 (most recently fully expanded leaf) were removed and bulked (x 4) for sodium and phosphorus analysis. Average daily PAR was 8.0 mol m^{-2} d^{-1}. Average daily glasshouse air temperature was 20.2°C (range: 13.5°C – 34.0°C), and average daily root solution temperature was 20.9°C (range: 18.7°C – 23.4°C). A second experiment (Exp 2) was completed using two *T. turgidum* lines (Line 227 and Line 161), Janz and Wollaroi (see Table 2.1), grown at 150 mM NaCl in modified ½ Hoagland's solution (containing 50 μM P) for 17 d. Four replicate seedlings per line were randomly spaced and harvested after 17 d in salt treatment. Leaves were separated into green leaf and dead leaf portions for phosphorus and sodium analysis and the percentage dead leaf (% DL) was calculated as the dry weight of dead leaf as a percentage of total leaf dry weight.

2.2.4 Screen for tissue tolerance to Na^+ in a collection of tetraploid landraces (Exp 3)

Forty seven tetraploid lines, 2 durum cultivars, 4 bread wheat cultivars and a barley cultivar were screened for symptoms of leaf injury when grown in 150mM NaCl with

10 mM $CaCl_2$ (Table 2.1). Current Australian durum cultivars Wollaroi and Tamaroi were included. Representative Australian bread wheat cultivars Janz, Machete and Westonia were also screened, together with the Indian landrace Kharchia, reputed to be salt-tolerant. Lines were randomly double spaced (one plant per alternate pot) within a block design. Entire shoots were harvested at 28 DAE, which corresponded to 3 weeks in 150 mM NaCl. Leaf blades were separated into green and dead leaf portions, dried (70°C for 3 d) and weighed. The percentage dead leaf was calculated as the weight of dead leaf as a percentage of total leaf weight. Immediately prior to harvest, leaf chlorophyll content was estimated using a chlorophyll meter (SPAD-502 meter, Minolta, Osaka, Japan). The SPAD-502 meter measures chlorophyll as the difference of optical density (OD) at two wavelengths, 650 nm and 940 nm. Mean leaf chlorophyll content for each genotype was derived from 3 readings taken at the base, middle and tip of leaf 1, 2 and 3 (most recent fully expanded leaf). Average daily PAR was 15.6 mol m^{-2} d^{-1}. Average daily glasshouse air temperature was 19.1°C, and average daily root temperature was 19.9 °C.

2.2.5 Assessment of glycinebetaine and proline levels due to salt stress (Exp 4)

Five tetraploid lines (Line 414, Line 528, Line 139, Line 255 and Line 362) were selected from Experiment 3, each representing the most tissue tolerant selection within a subspecies. These selections were compared with durum cultivars, Wollaroi and Tamaroi and barley cultivar Skiff. A Na^+-excluding tetraploid landrace, Line 149, was also included (Munns et al., 2000b). Seedlings were randomly doubly spaced in trays and grown in 150 mM NaCl with 10 mM $CaCl_2$ and modified ½ Hoagland's solution (50 μM P) as described previously. The date of the appearance of the tip of leaf 3 above the sheath of leaf 2 was recorded for every plant. Typically this occurred between 8-11 DAE. The blade of leaf 3 was harvested 10 days after appearance. Leaf 3 blades from 3 seedlings were combined to produce one bulked replicate, 5 replicates per line were harvested, snap frozen in liquid N_2, freeze dried and finely ground for subsequent glycinebetaine and proline analysis. Leaf 3 from five additional plants were harvested at midday to measure Na^+, K^+ and Cl^- concentrations and osmotic potential. Average daily PAR was 11.5 mol m^{-2} d^{-1}. Average daily glasshouse air temperature was 20.7°C (day) and 14.5°C (night), and average daily root temperature was 18.2°C (day) and 16.6°C (night).

2.2.6 Analysis of glycinebetaine and proline

Metabolites were extracted from 100 mg samples of finely ground leaf tissues using 3 ml of ice-cold 5 % (v/v) perchloric acid, twice (Fan et al., 1993). The neutralised extracts were passed through a 0.22 μm filter prior to injection into the HPLC. The HPLC (Waters Corporation, Milford, MA, USA) consisted of a 600E pump, 717 auto-sampler, 996 photodiode array (PDA) detector and Millennium software. The method used was based on Naidu (1998). A Sugar-Pak column (300 mm length x 6.5 mm i.d.) with guard-insert (Waters Corporation, Milford, MA, USA), and mobile phase of 5 mg L^{-1} Ca-EDTA in Milli-Q water, were maintained at 85 – 90°C. The mobile phase was de-gassed prior to use, and the reservoir was sparged with He at 20 mL min^{-1}. Flow rate through the column was 0.6 mL min^{-1}. The sample injection volume was 50 μL. The PDA detector measured absorbance at 1.2 nm intervals between 193 nm to 300 nm, and quantification was at 195 nm. Peak purity and identity were determined by comparing the retention times and spectra of sample peaks to those of authentic standards.

2.2.7 Other chemical analyses

In Experiments 1 and 2, phosphorus and sodium were analysed on dried (70°C for 3 days), finely ground and pelleted leaf material using an X-ray fluorescence spectrometer (Phillips PW 1404, Eindhoven, The Netherlands) according to the method described by Norrish and Hutton (1977).

For Na^+ and K^+ analysis in Experiments 3 and 4, harvested leaf blades were rinsed with deionised water, dried at 70°C for 3 days, weighed and extracted in 500 mM HCl at 80°C for 1 h and analysed using an atomic absorption spectrophotometer (SpectrAA-300, Varian, Melbourne Australia).

Osmotic pressure (π) was measured on leaves that were snap frozen in liquid N_2 and stored at -20°C until analysis. Leaves were thawed, the sap was squeezed out between folds of Nescofilm, centrifuged for 1 min at 14 000 rpm to remove particles, and then the osmolality was measured with a freezing point depression osmometer (Micro-Osmometer Model 3MO, Advanced Instruments Inc., Needham Heights, MA, USA). Osmotic pressure (MPa) was calculated as cRT, where RT is 2.48 (litre-MPa per mole) and c is concentration (osmol kg^{-1}).

2.2.8 Data analysis

Data from Experiments 3 and 4 were analysed using analysis of variance, and LSDs (P=0.05) were used to compare genotype means.

2.3 RESULTS

2.3.1 Effect of salinity and phosphorus on leaf injury

To ascertain whether the leaf death of plants grown in saline conditions was due to P toxicity rather than Na^+ toxicity, six tetraploid genotypes lines with contrasting degrees of Na^+ accumulation were compared. Plants were grown with and without NaCl in full strength Hoagland's solution containing the standard P concentration of 1 mM P. Figure 2.2 shows the relationship between leaf death and P concentration of leaves from seedlings grown in control or salt treatment.

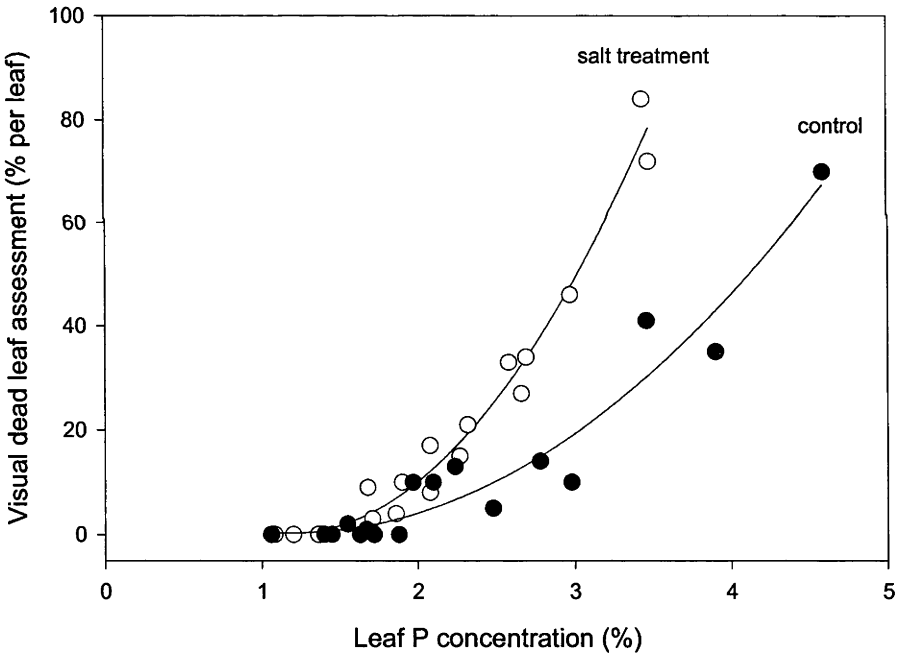

Figure 2.2 Relationship between leaf P concentration and leaf injury assessed visually in leaves from control (●) and salt-treated (○) tetraploid seedlings grown in control or 150 mM NaCl for 17 d. Values for dead leaf assessment are means (n = 9). The curves fitted to the data described by the following polynomial equations, respectively: (salt) $y = 21.3 - 35.4x + 14.9x^2$ ($r^2 = 0.98$); (control) $y = 8.7 - 14.0x + 5.8x^2$ ($r^2 = 0.93$).

There was genotypic variation in P uptake, with a 2 to 3 fold range in both control and salt conditions (data not shown). Leaf death was greater in lines with higher P uptake and was unrelated to leaf Na^+ toxicity. Leaf death and P concentrations above 1.8% had a high correlation ($r^2 = 0.93$ for the control and $r^2=0.98$ for the salt treatment),

but the slope was much greater for the salt than the control treatment. These results indicate that P concentrations greater than about 1.8% in leaves was toxic, causing leaf death. Na^+ concentrations in control leaves were always less than 200 μmol g^{-1} DW and in the salt-treated leaves ranged from 110 to 1250 μmol g^{-1} DW, but there was a poor correlation ($r^2 = 0.09$) between the Na^+ concentration and leaf death (Appendix 2.3).

The results indicate that salinity increased the sensitivity of these lines to P, causing leaf death at lower P levels than for those in the control conditions. For example, approximately 3% P resulted in 50% leaf death in salt conditions whereas about 4% P resulted in the same degree of leaf death in control conditions (Figure 2.2). Therefore P toxicity was masking leaf responses to high Na^+.

To evaluate the impact of reducing the P concentration in the nutrient media on leaf injury, four (of the six) genotypes (Table 2.1) were grown in 150 mM NaCl in ½ Hoagland's solution with P at a concentration of 50 μM (Exp 2). P concentrations were about 0.6% P in both dead leaf and green leaf material (Table 2.2). These low P concentrations in both dead and green leaf material indicated that P was not responsible for leaf death in this experiment. Rather it was the high Na^+ concentrations, which were between 1.5 – 4.2 mmol gDW^{-1} (equivalent to 250 – 700 mM on a tissue water basis) in dead leaf material. 0.6% P is considered an optimal level for wheat leaves (Reuter and Robertson, 1986), and therefore ½ modified Hoagland's solution containing only 50 mM P was used in subsequent experiments.

Table 2.2 Na^+ concentration (mmol gDW^{-1}), phosphorus content (%) and percentage dead leaf (% DL) of four wheat genotypes differing in Na^+ concentration when grown in 150 mM NaCl in modified ½ Hoagland's solution (50 μm P) for 17 d. Values are means from bulked samples (n=4).

Genotype	Na^+ concentration (mmol gDW^{-1})		% DL	Phosphorus content (%)	
	Green leaves	Dead leaves		Green leaves	Dead leaves
Janz	0.32	2.93	9.4	0.57	0.54
Wollaroi	0.52	2.87	11.2	0.54	0.66
Line 227	0.97	4.20	8.3	0.63	0.60
Line 161	1.14	1.46	12.6	0.61	0.54

2.3.2 Screen for tissue tolerance to Na^+ in a collection of tetraploid landraces

Forty seven tetraploid lines, 2 durum cultivars, 4 bread wheat cultivars and a barley cultivar were screened for tissue tolerance to Na^+. Four methods were tested to assess variation in leaf injury due to a high external salt concentration; 1) percent dead leaf (weight of dead leaf as % of total leaf weight), 2) the ratio between the percent of dead leaf and the total leaf Na^+ content, 3) the Na^+ concentration in the dead leaf, and 4) the chlorophyll content of the three oldest leaves. The first two methods provided direct measurements of leaf injury and the relation to Na^+ uptake, but were destructive of the whole plant. The second two methods were indirect or incomplete estimates, but were not destructive. Barley was included as a benchmark, because of its established reputation for salinity tolerance coupled with high rates of salt accumulation, and previous observations that it was slow to develop leaf injury. The results of these measurements are summarised in Table 2.3 (values for all lines are in Appendix 2.4).

1) Percent dead leaf

Significant variation in percent dead leaf (weight of dead leaf as % of total leaf dry weight) was found among lines, the percent dead leaf ranging from 2 to 28 % (Figure 2.3 and Table 2.3).

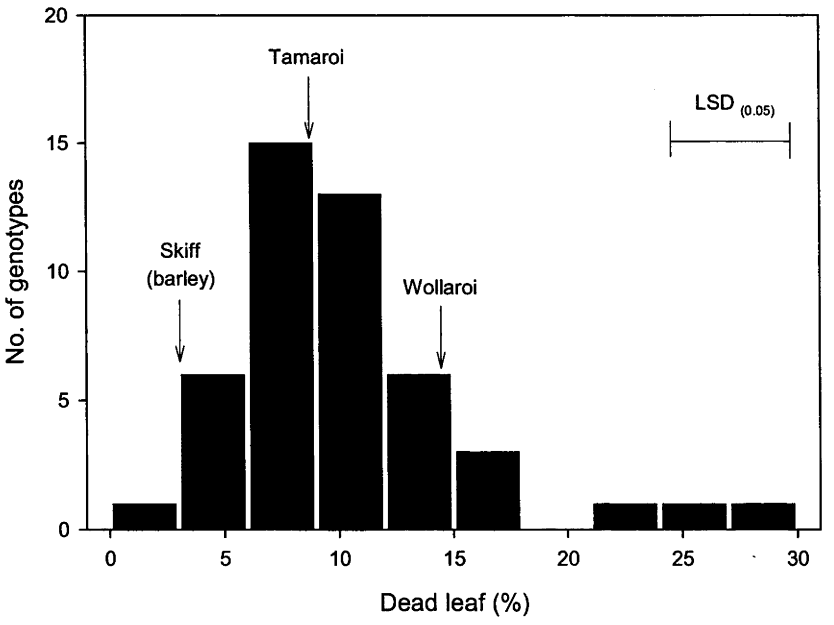

Figure 2.3 Frequency distribution of percentage dead leaf of 47 tetraploid wheat selections, grown in 150 mM NaCl for 21 d. The bar represents a LSD (P=0.05) for among line comparisons.

Table 2.3 Mean and range of percentage dead leaf (% DL), the ratio of % DL to total leaf Na^+, Na^+ concentration in dead leaf and mean chlorophyll estimate (leaves 1, 2 and 3 on main stem) in different subspecies of *T. turgidum*, durum wheat cultivars, bread wheat cultivars and a barley cultivar grown in 150 mM NaCl for 21 d. Selections from each subspecies with the lowest % DL are also listed.

Lines or cultivars	Parameter or cultivar name	%DL (%)	Ratio of %DL to leaf Na^+ content (mmol)	Na^+ concentration in dead leaf (mmol gDW^{-1})	Mean chlorophyll of leaves 1,2 & 3 (SPAD units)
Ssp. durum selections	Min	5.1	17	1.46	12.2
	Max	15.2	67	4.68	35.2
	Mean(n=17)	10.1	26	3.72	22.1
	Line 139	5.1	24	3.94	30.8
Ssp. polonicum selections	Min	4.2	11	3.81	17.3
	Max	10.3	21	5.11	31.5
	Mean(n=11)	7.8	16	4.29	20.9
	Line 255	4.2	11	3.81	31.5
Ssp. turgidum selections	Min	5.6	11	3.60	16.0
	Max	12.5	36	4.32	29.9
	Mean (n=7)	9.0	17	3.94	23.2
	Line 362	5.6	11	3.69	29.9
Ssp. carthlicum selections	Min	2.2	9	2.97	10.7
	Max	28.4	59	4.23	32.7
	Mean (n=6)	18.1	23	3.54	20.1
	Line 414	2.2	9	3.87	32.7
Ssp. turanicum selections	Min	6.0	15	2.52	19.7
	Max	13.2	37	3.69	36.6
	Mean (n=6)	10.1	26	3.31	24.5
	Line 528	6.0	15	2.52	36.6
Durum wheat cultivars	Wollaroi	14.5	71	2.86	13.9
	Tamaroi	8.4	27	4.30	28.7
Bread wheat cultivars	Janz	8.0	44	2.97	19.9
	Machete	7.6	48	3.83	20.7
	Westonia	6.6	45	3.90	23.6
	Kharchia	5.8	31	3.57	23.6
Barley cultivar	Skiff	3.0	9	4.08	31.2
	$LSD_{(0.05)}$	5.5	18	1.06	9.1

The barley cultivar Skiff had a low degree of leaf injury, as expected (only 3 % of leaves were dead). Bread wheat cultivars and the durum cultivar Tamaroi had a relatively low percent dead leaf (6-8 %) but the durum cultivar Wollaroi was higher (15 %). There was a 2 – 3 fold range in percent dead leaf in four of the tetraploid subspecies, and a 13 fold range in ssp. *carthlicum*. The ssp. *carthlicum* selection Line 414 had a low degree of leaf injury, at least as low as barley (Table 2.3).

2) Ratio between percent dead leaf and leaf Na^+ content

The ratio of % dead leaf to Na^+ content (whole shoot basis) was calculated as an index of tolerance to Na^+ in the leaves. A lower % dead leaf per Na^+ content might indicate a higher degree of tissue tolerance to Na^+. The total leaf Na^+ content of individual genotypes did not correlate with the % dead leaf (Figure 2.4), suggesting there might be genotypic variation in the ability to tolerate the Na^+ at the tissue or cellular level. This

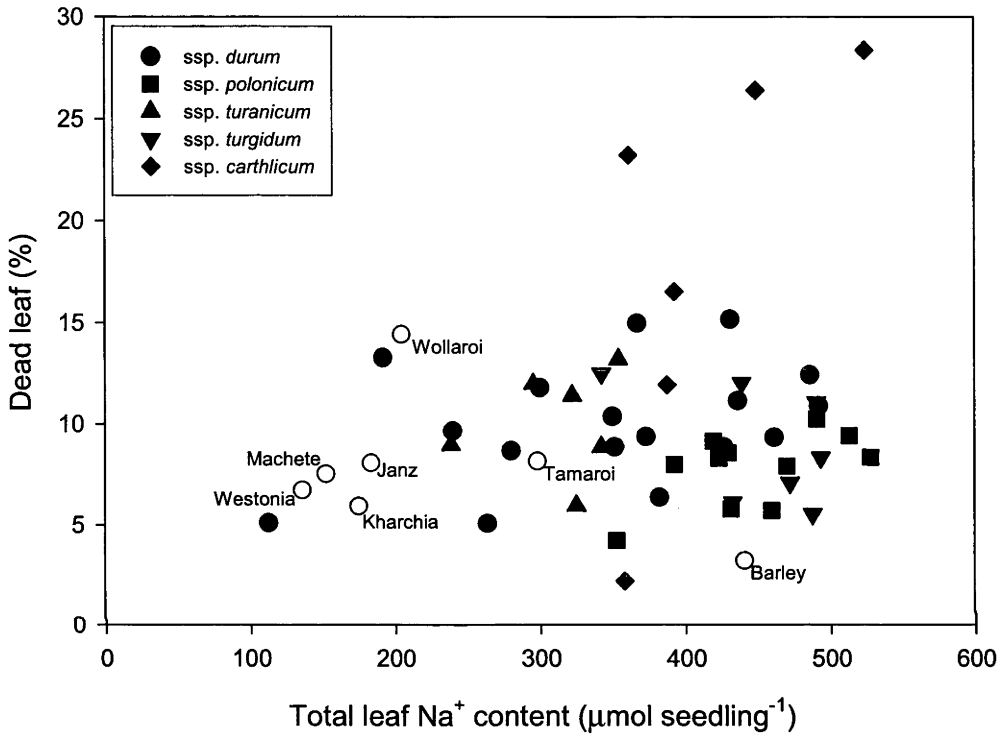

Figure 2.4 Relationship of total leaf Na^+ content and percentage dead leaf (% DL) of 47 tetraploid wheat selections, 4 hexaploid wheat cultivars (Westonia, Janz, Machete, Kharchia), 2 durum cultivars (Tamaroi, Wollaroi) and a barley cultivar (Skiff), grown in 150 mM NaCl for 21 d.

ratio of % dead leaf per Na^+ content ranged from 9 – 71, with Skiff barley at the low end of that range with a value of 9 (Table 2.3, Figure 2.5). Analysis of variance showed that there were significant differences (P= 0.05) between a number of tetraploid selections and both Wollaroi and Tamaroi. The selections with the lowest % dead leaf in 4 of the 5 subspecies also had the lowest ratio of % dead leaf to leaf Na^+ content, similar to that of Skiff (Table 2.3). The bread wheat cultivars, however, while excluding 2 – 3 times the amount of Na^+ from the leaves, displayed similar levels of leaf injury to a number of tetraploid selections, indicating greater sensitivity to tissue Na^+ levels (Figure 2.4, Table 2.3).

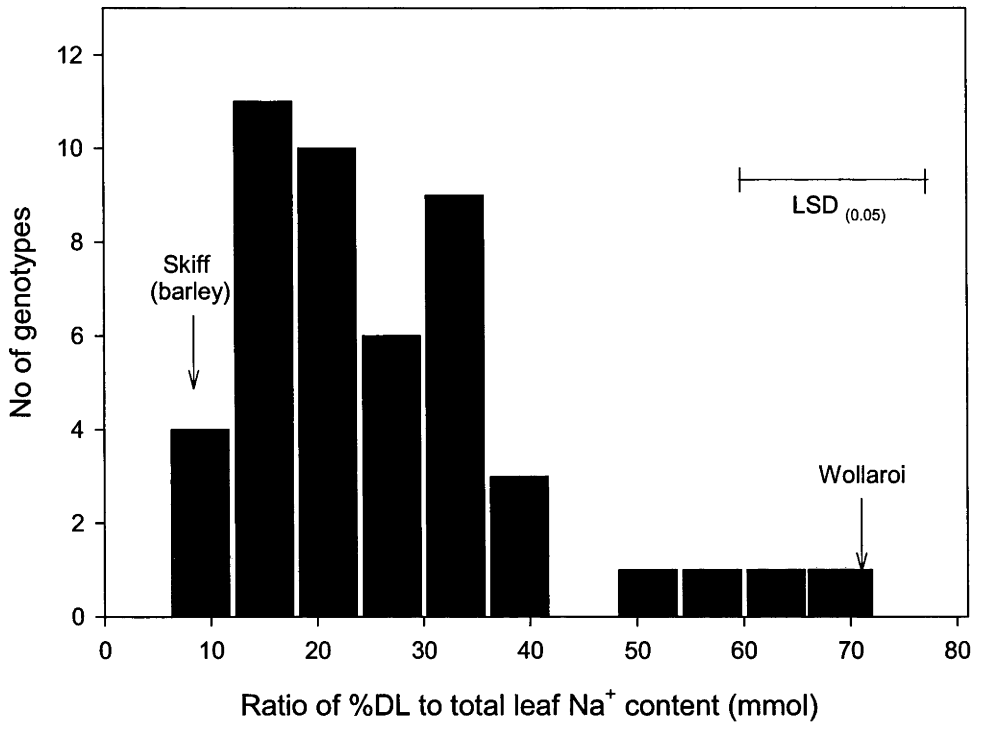

Figure 2.5 Frequency distribution of the ratio of percentage dead leaf to leaf Na^+ content of 47 tetraploid wheat selections, grown in 150 mM NaCl for 21 d. The bar represents LSD (P=0.05) for among line comparisons.

3) Na^+ concentration in dead leaf

The Na^+ concentration in the dead leaf material was measured as a possible non-destructive tissue tolerance parameter, as a higher Na^+ concentration in dead leaves may indicate a greater tolerance threshold prior to leaf death. However, there was little genotypic variation in Na^+ concentration in the dead leaf material (Table 2.3). Further, selections from each subspecies identified as more tissue tolerant using other screening parameters (% DL and % DL per leaf Na^+ content – Table 2.3) did not contain the highest Na^+ concentration in the dead leaf, and while higher than Wollaroi, were not statistically different.

4) Chlorophyll content

Chlorophyll concentration (estimated with a SPAD meter) of the three oldest leaves on the main stem was measured to determine if there was a relationship between this simple non-destructive measure of leaf injury and the total plant injury measured with a destructive harvest. Selections having the lowest total % dead leaf also had the highest

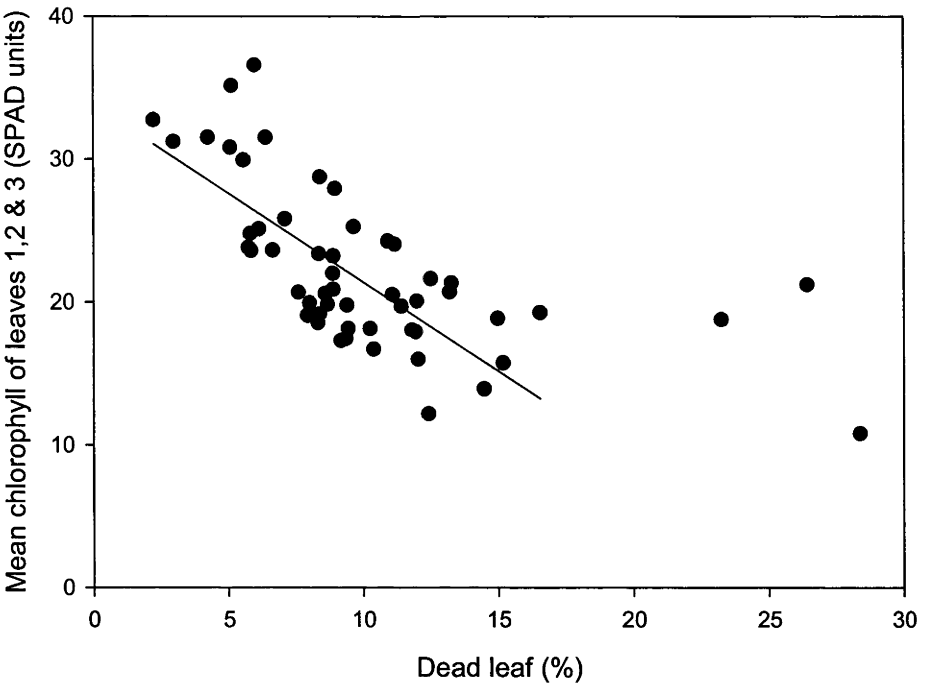

Figure 2.6 Relationship between percentage dead leaf and mean chlorophyll content of main stem leaves 1, 2 and 3 of 47 tetraploid wheat selections, grown in 150 mM NaCl for 21 d. A linear regression line is fitted for all genotypes with less than 20% dead leaf. Fitted regression is described by following equation; $y = -3.73(x) + 101.4$ ($r^2 = 0.51$).

chlorophyll estimates for leaves 1-3 (Figure 2.6). Further, selections from four of the five tetraploid subspecies with the lowest % dead leaf also had the highest mean chlorophyll content (Table 2.3).

There was a moderate correlation between mean chlorophyll content and % dead leaf ($r^2 = 0.51$). Three particularly sensitive landraces from the *carthlicum* ssp. with high degree of leaf death (20 – 30 %) did not fit on the linear regression comparing chlorophyll content with % DL. This was due to substantial leaf death also occurring on leaves 4 and 5 on the main stem and on tiller leaves, which increased % dead leaf of whole seedlings but not the chlorophyll content of main stem leaves 1, 2 and 3.

2.3.3 Genotypic variation in glycinebetaine and proline levels due to salt stress

To examine the contribution of compatible solutes to salt tolerance, with the aim of evaluating their use as a selection tool for the trait of tissue tolerance, glycinebetaine and proline were measured in 9 lines; five candidate tissue tolerant tetraploid lines (selected from Experiment 3), three genotypes with high leaf Na^+ (Skiff barley, and the durum cultivars Tamaroi, and Wollaroi, which in Experiment 2 showed leaf death ranging from 2 to 15%), and the low Na^+ tetraploid genotype, Line 149.

The concentration of glycinebetaine in leaf 3 of all genotypes increased by 6 – 18 fold due to salt stress (Table 2.4). Significant genotypic variation existed between salt-treated lines with barley showing the lowest concentration at 6 mM on a leaf water basis (corresponding to 39 μmol g^{-1} DW) and the low Na^+ Line 149 the highest at 15 mM (corresponding to 69 μmol g^{-1} DW). Most tetraploid selections were not different to durum cultivar Wollaroi and all were less than durum cultivar Tamaroi.

Table 2.4 The effect of salinity on the accumulation of glycinebetaine, Na^+ concentration, leaf osmotic potential ($\Psi\pi_{salt}$) and the calculated contribution of glycinebetaine to the osmotic potential (salt treatment) in two durum cultivars, a barley cultivar and six tetraploid selections. FW:DW ranged from between 5.5 to 7.5 in salt treatment and 7.4 to 9.3 in control. All measurements were on leaf 3 after 10 d in treatment. Least significant differences (LSDs) are provided for among genotype comparisons.

Genotypes	Glycinebetaine (mM)			Na^+	$\Psi\pi_{salt}$	$\pi_{glycinebetaine}$[b]
	Control	Salt treatment		concentration		(cytoplasm)
	leaf	leaf	cytoplasm[a]	(mM)	(MPa)	(% of $\Psi\pi_{salt}$)
Durum cultivars:						
Wollaroi	0.5	7.9	39.4	196	-2.10	4.7
Tamaroi	0.8	10.7	53.7	206	-1.91	7.5
Barley cultivar:						
Skiff	0.4	6.0	30.0	242	-1.91	3.9
Tetraploid lines:						
Line 362	1.3	8.1	40.3	244	-2.09	4.8
Line 255	1.0	8.4	42.2	220	-2.07	5.1
Line 139	0.7	7.2	36.1	243	-2.10	4.3
Line 528	0.4	7.4	37.0	235	-1.86	5.0
Line 414	1.5	9.9	49.3	240	-2.10	5.9
Low Na^+ line:						
Line 149	1.7	15.1	75.8	47	-1.83	10.4
LSD $_{(0.05)}$	0.2	1.1	5.4	45	0.27	0.9

[a]glycinebetaine concentration in the cytoplasm was calculated on the assumption that the cytoplasm (cytosol and chloroplasts) constitutes 20% of the total cell water fraction and that glycinebetaine only accumulates in the cytoplasm.

[b]the osmotic potential of glycinebetaine ($\Psi\pi_{glycinebetaine}$) was calculated on the assumption that glycinebetaine is contained only within the cytoplasm.

No relationship appeared between leaf injury estimated as % dead leaf (Table 2.3) or Na^+ concentration of leaf 3 (Table 2.4) and glycinebetaine levels for the same selections. In fact, the genotype with the lowest Na^+ concentration (Line 149) had the

highest glycinebetaine level in both the control and salt treatment. Proline could not be detected within the limits of the analysis (2 μmol gDW^{-1}), even in the salt-treated plants.

Based on the assumption that the cytoplasm (cytosol and chloroplasts) accounts for about 20% of the total leaf aqueous volume (Harvey and Thorpe, 1986; Winter at al., 1993) and that glycinebetaine preferably accumulates in the cytoplasm (see summary by McCue and Hanson, 1990), genotypic variation in the concentration of glycinebetaine in the cytoplasm ranged between 30 to 76 mM, which accounted for 5 to 10 % of the total osmotic pressure (in the cytoplasm) in the salt-treated genotypes (Table 2.4).

2.4 DISCUSSION

2.4.1 Variation for Na^+ tissue tolerance in tetraploid wheat

High internal Na^+ levels were identified in many landraces from various *T. turgidum* subspecies. Five of these landraces maintained a high percentage of green healthy leaves despite having high levels of Na^+, similar to barley cv. Skiff (Table 2.3, Figure 2.3), indicating that they may have the ability to tolerate high internal Na^+ at the tissue or cellular level. Significant genotypic variation existed in each *T. turgidum* ssp. for tissue tolerance indicators, % DL and the ratio of % DL to leaf Na^+ content. Lines from ssp. *polonicum* had on average a higher degree of tissue tolerance than other subspecies. Lines from ssp. *carthlicum* had the highest mean % DL and highest mean ratio of % DL to leaf Na^+ content, indicating that this subspecies contained fewer tissue tolerant genotypes than the others. A previous study by Munns et al. (2000b) showed that lines from ssp. *carthlicum* had on average, higher leaf Na^+ concentrations than other *T. turgidum* ssp. This may have in part accounted for the lower degree of tissue tolerance, as very high Na^+ concentrations may have led to a rapid breakdown of cellular partitioning or vacuolar compartmentation ability. Apparently, little is known about any defining physiological features relating to salt tolerance of the *T. turgidum* ssp; the classification of landraces within each ssp. based largely on head morphology characteristics rather than geographical location (Ray Hare pers. com). It was therefore not surprising that tissue tolerant lines occurred in all *T. turgidum* subspecies, and not just one.

2.4.2 Assessment of screening methods

Of the two non-destructive methods tested, only the chlorophyll concentration of main stem leaves 1, 2 and 3, estimated with a SPAD meter, correlated well with % dead leaf (in lines established as having high Na^+ accumulation) and the ratio of % DL to leaf Na^+ content. This method appears to be an adequate surrogate for measuring extremes in % dead leaf and therefore could provide a useful screening tool when assessing tissue tolerance in breeding populations. The accuracy of this method, however, would be dependent on the duration of the screen, with enough time allowed to observe genotypic differences in chlorophyll degradation on the older main stem leaves, without substantial leaf death occurring on other leaves.

The Na^+ concentration in the dead leaf material was measured on the presumption that tissue tolerant selections might tolerate unusually high Na^+ concentrations prior to leaf death. Results from these measurements, however, did not correlate well with other three screening methods and there was little genotypic variation in Na^+ concentration of dead leaves. As some leaves may have died a week or more before harvest, it is possible that the lack of variation in Na^+ concentration may be due to contamination or alternatively, there may have genotypic differences in stress-induced premature leaf senescence.

The screening method that relates shoot Na^+ concentration to leaf injury or death (ratio of % DL to leaf Na^+ content) is likely to be the best indicator of Na^+ tissue tolerance, as it accounts for even small genotypic differences in Na^+ accumulation which may cause leaf death. Additionally, leaf death assessed in isolation could be due to a number of factors (Section 2.4.3). In the only other screen of this kind, Yeo et al. (1990) using a similar criteria (Na^+ in leaf corresponding to 50% chlorophyll loss) found a five fold range in tissue tolerance amongst 21 rice accessions. Interestingly, these authors found that tissue tolerance in rice was inversely correlated with vigour and also that the taller traditional varieties were more sensitive, compared to the shorter dwarf varieties. In the current study, the five landraces identified with the highest degree of tissue tolerance were on average, twice the height of Wollaroi, which was one of the most sensitive lines. This example emphasises the potential difficulties of comparing cultivars with landraces in the growth experiments. Floral initiation often occurs earlier in cultivars than in the landraces, even when both are vernalised. The earlier shift from leaf area production to stem elongation in the cultivars means that leaf

and tiller production are curtailed (affecting parameters such as leaf death as a percentage of total leaf) and also means that the growth rates (biomass production) of the two types can no longer be compared. Feasible screening methods for traits such as tissue tolerance should also take into account pleiotropic or interactive effects of other variables such as genetic differences in growth rate, morphology or phenology.

2.4.3 Factors affecting leaf injury which are not related to tissue tolerance

As tissue tolerance to high leaf Na^+ concentrations cannot be measured directly, indirect ways were explored, such as measuring injury that might occur as a result of lack of compartmentation of salts. However, leaf injury could arise from a number of reasons. First there could be the osmotic effects of salt in the soil solutions, causing accelerated senescence due to leaf water deficit or hormonal effects arising from root signals (Termaat et al., 1985). Second, there could be nutrient imbalances resulting in deficiencies or excesses of other ions (Greenway and Munns, 1980). Third, there could be toxic effects of salts in the leaves, due to excessive salt build up in cytoplasm or cell wall. It is only the last effect that is relevant to compartmentation of Na^+ and hence tissue tolerance, but it is difficult to separate from the other effects. Hence, it is essential to know what the cause of the injury is if the selected germplasm is to be used in a breeding program.

This issue was highlighted in my experiments by the finding that high P (in the nutrient solution), particularly in conjunction with salinity, caused a greater degree of P accumulation in leaves and a high degree of leaf death, not associated with Na^+ toxicity. A similar a trend of higher leaf P was found in four rice lines grown in 50 mM NaCl compared to zero salt controls (Aslam et al., 1996). It appears that the presence of higher salt concentrations in the leaves may have affected the transport and partitioning of P (perhaps leading to higher P concentrations into cell walls) leading to accelerated leaf death (Nieman and Clark, 1976). The issue of salinity-induced P toxicity is likely to only occur in experiments conducted in sand or hydroponics, due to higher P concentrations in solution cultures compared to soil solutions and the greater availability of P in solution cultures compared to soil (summarised by Grattan and Grieve, 1999).

Similarly, nitrogen (N) nutrition is another likely complicating candidate. N deficiency will cause accelerated leaf senescence due to demand for N to be remobilised from old leaves. Conversely, under some circumstances, excess N can cause leaf burn.

2.4.4 Glycinebetaine, proline and Na^+ tissue tolerance

The concentration of glycinebetaine of all selections increased substantially due to salt stress (Table 2.4). This extent of increase was similar to that observed in hexaploid wheat (Colmer et al., 1995; Sabry et al., 1995), barley (Wyn Jones and Storey, 1978b) and spinach (Robinson and Jones, 1986). Organic solutes such as glycinebetaine and proline might be high in the genotypes with tissue tolerance, as they could be required in the cytoplasm to balance the osmotic pressure generated from the storage of high concentrations of Na^+ and Cl^- in the vacuole. There was little variation in leaf Na^+ concentration between most genotypes and therefore it was difficult to determine a relationship between glycinebetaine and Na^+ concentration. The highest glycinebetaine levels were found in Line 149, a low Na^+ tetraploid line, which accumulated 4 – 5 times less Na^+ than the remaining genotypes. This result is consistent with Colmer et al. (1995) who found the highest betaine levels in the youngest wheat leaves with the lowest Na^+ concentrations. Further, glycinebetaine levels of salt stressed plants were not related to the degree of leaf injury. The putative tissue tolerant tetraploid lines contained similar levels of glycinebetaine to Wollaroi despite a 2 to 6 fold difference in % dead leaf (Table 2.3).

It was calculated that glycinebetaine made only a small contribution to osmotic adjustment, even if it was all present in the cytoplasm. Glycinebetaine (in the cytoplasm) contributed only 5 – 10% of the total osmotic potential of the leaf. This was similar to that of the wheat variety 'Chinese Spring' (~ 6 %), measured by Colmer et al. (1995), which contained similar concentrations of glycinebetaine to the genotypes surveyed in the current study. Other organic osmotica such as sugars, amino acids other than proline (see below), and inorganic osmotica such as K^+ presumably played a much more substantial role in the cytoplasm in balancing the high osmotic pressure in the vacuoles, that would result from the sequestration of most of the Na^+ and Cl^- into that compartment. K^+ accounted for about 20% of the total leaf osmotic potential (Appendix 2.5). If, however, only half of the K^+ was partitioned to the cytoplasm, this would account for about 80% of the total osmotic potential of that compartment.

Proline could not be detected within the limits the analysis technique used (2 μmol gDW^{-1}), even in the salt-treated plants. This is not surprising as the stress imposed by the salinity treatment was relatively small, resulting in only a 50% growth reduction after several weeks, and previous work on drought-stressed wheat had shown that proline started to accumulate markedly only after shoot growth was severely inhibited (Munns et al., 1979). Other studies showed proline to increase in salt-affected wheat (Colmer et al., 1995; Sabry et al., 1995), and increase with increasing leaf Na^+ concentrations in older leaves (Colmer et al., 1995) but the salinity used was higher than in this study.

In summary, there was no detectable accumulation of proline and while significant genetic variation in glycinebetaine accumulation under salt stress was found, there was no genotypic correlation with either Na^+ accumulation or leaf injury, indicating that glycinebetaine accumulation is not likely to be a useful screen for tolerance to high leaf Na^+ concentrations in tetraploid wheat.

2.4.5 Conclusions

The criteria used to screen for tissue tolerance in these experiments were quite time consuming, required destructive harvests and costly analytical techniques. Rapid, simple and preferably non-destructive screening methods will be needed for efficiently screening large germplasm collections, for genotype selection, for developing molecular markers and for pyramiding traits or genes.

Understanding the physiology of the traits conferring salinity tolerance will form the basis for further improvements in screening for salinity tolerance of agricultural species. With respect to Na^+ tissue tolerance, the relationship between leaf Na^+ concentration and physiological performance (eg. photosynthesis) in salt stress plants needs to be assessed, to determine what constitutes a toxic Na^+ concentration in the leaf.

Chapter 3

FACTORS AFFECTING CO_2 ASSIMILATION, LEAF INJURY AND GROWTH IN SALT-STRESSED DURUM WHEAT

3. FACTORS AFFECTING CO_2 ASSIMILATION, LEAF INJURY AND GROWTH IN SALT-AFFECTED DURUM WHEAT

3.1 INTRODUCTION

The capacity of plants to tolerate high levels of salinity depends on the ability to exclude salt from the shoot, or to tolerate high concentrations of salt in the leaf. While most halophytes depend on a combination of both mechanisms to survive in extremely saline environments, non-halophytes and in particular, most crop species have mainly been characterised as salt-tolerant or salt-sensitive on the basis of Na^+ exclusion capability. Durum wheat (*Triticum turgidum* L. ssp *durum* Desf.) typically has high rates of Na^+ accumulation and has been found to be more salt-sensitive than bread wheat (e.g. Rawson et al., 1988b). The lower salt tolerance of durum than bread wheat is considered to be due to these high rates of Na^+ accumulation in the leaves and poor K^+/Na^+ discrimination (Gorham et al., 1987). Recently, novel tetraploid germplasm has been identified with low Na^+ accumulation and high K^+/Na^+ discrimination, which has the potential for improving salt tolerance of durum wheat cultivars through conventional breeding (Munns et al., 2000b).

Although the degree of salt tolerance has been found to correlate inversely with Na^+ accumulation in the leaves of barley (Greenway, 1962), rice (Flowers and Yeo, 1981), olives (Tattini et al., 1992) and in diploid wheat (Schachtman et al., 1991), Schachtman et al. (1991) found that Na^+ concentrations did not always predict the salt tolerance of all *Triticum tauschii* accessions. Similarly, in rice (Yeo and Flowers, 1983) and maize (Cramer et al., 1994b), salt tolerance of some individuals did not correlate with leaf Na^+ concentrations, indicating variation in the degree of cellular or tissue tolerance to Na^+. Tissue tolerance depends largely on the capacity to compartmentalise salt ions into safe storage places such as vacuoles. This mechanism therefore prevents both dehydration when salts might build up in cell walls, and the breakdown of key physiological processes such as photosynthesis through membrane damage or enzyme inhibition when salts may build up to toxic levels in the cytoplasm (Greenway and Munns, 1980; Munns, 1993). Genetic variation for tissue tolerance to high internal salt concentrations in both durum wheat and hexaploid (bread) wheat is unknown.

Traditional screening techniques have used agronomic selection criteria such as survival, biomass accumulation and yield to investigate genotypic differences in salt tolerance. There are many inherent problems with this approach such as the heterogeneity of saline fields, differential growth and developmental patterns between genotypes and species, and logistical and time constraints with long-term growth comparisons. In recent years, the focus in screening has shifted towards examining specific physiological mechanisms involved in salt tolerance and devising appropriate simple, quick, and accurate techniques to reliably assess genotypic variation in salt tolerance. Measuring chlorophyll fluorescence is a good example of this approach, as it monitors the function of the photosynthetic apparatus through a simple and non-destructive process. A number of studies have attempted to utilise fluorescence parameters to examine factors limiting photosynthesis of salt-affected plants (e.g. Brugnoli and Björkman, 1992), to compare salt-treated and control plants (e.g. Belkhodja et al., 1994; Shabala et al., 1998) or to differentiate between salt-tolerant and salt-sensitive genotypes (e.g. Lutts et al., 1996). Screening and selection on the basis of individual physiological mechanisms also has the potential of creating even greater salt tolerance, by combining or pyramiding traits, provided that they are easily distinguishable, stable and heritable in a breeding program (Yeo and Flowers, 1986; Noble and Rogers, 1992).

The objectives of this study were twofold. Firstly, I aimed to investigate the factors affecting CO_2 assimilation and leaf injury, with a particular focus on the relationship between ion content with gas exchange, chlorophyll fluorescence, and chlorophyll degradation. Two tetraploid wheat genotypes that differed in leaf injury were used in this study. Previous experiments showed substantially less leaf injury associated with a tetraploid landrace 'Line 255' than in the durum cultivar 'Wollaroi', while each had similar leaf Na^+ concentrations when grown under glasshouse conditions in 150 mM NaCl (Table 2.3). Secondly, I aimed to identify physiological variables that could be used to develop a screening technique that would differentiate between wheat genotypes tolerant and sensitive to high internal concentrations of salt.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growth conditions

Two durum wheat genotypes with contrasting degrees of leaf injury for a given Na^+ concentration were used in this study; *Triticum turgidum* L. ssp. *durum* Desf. cv Wollaroi (current cultivar widely grown in eastern Australia) and a tetraploid wheat landrace; Line 255 (*Triticum turgidum* L. ssp. *polonicum*). Seeds of Line 255 were provided by Dr Ray Hare, of the Centre for Crop Improvement, NSW Agriculture, Tamworth NSW.

Seeds of Wollaroi and Line 255 were selected by weight (55 – 65 mg), surface sterilised with 1% hypochlorite, and germinated in Petri dishes for 3 d. Germinated seeds were planted (one plant per pot) into 6.5 x 15.8 cm pots containing coarse quartz gravel, in a 90 L plastic moulded tray containing 153 pots. Seedlings were randomly double spaced to avoid problems with crowding. The tops of the pots were covered with a rigid white plastic sheet with openings for the emerging seedlings, to prevent salt contact of lower leaves from the quartz gravel. Seedlings were watered using an automatic subirrigation system (Munns et al., 1995), whereby solutions were pumped into trays and then drained into holding tanks every 30 min. For the salt treatment, seedlings were watered with half strength modified Hoagland's solution (P concentration reduced from 1 mM to 100 μM), then at 6 d after emergence (DAE), 25 mM NaCl was added twice daily over 3 d to a final concentration of 150 mM. Supplemental Ca^{2+} was also added as $CaCl_2$ to give a final Na^+: Ca^{2+} of 15:1. For the control treatment, seedlings were watered with half strength modified Hoagland's solution. All solutions were changed fortnightly and pH and electrical conductivity monitored each week

The plants were grown in a controlled environment chamber with a 9 h photoperiod and a maximum PPFD of 1150 μmol m^{-2} s^{-1}, provided by 12 1000-W metal arc and 24 60-W incandescent lamps. Light intensity reflected up from the white covers (described above) was measured at about 600 μmol m^{-2} s^{-1}. The average air temperature was 22.7°C (range: 20.4 – 24.6°C) during the day and 19.2°C (range: 17.4 – 21.0°C) during the night. Average root temperature was 19.7°C (range: 18.6 – 21°C) during the day and 19.6°C (range: 18.8 – 20.4°C) during the night. Control and salt treatments were carried out sequentially in the same chamber. Light and temperature were carefully

matched for each treatment. Harvests of both genotypes were made at six times, 18, 20, 25, 28, 32 and 35 DAE, which corresponded to between 7 and 26 d after appearance for leaf 3. For each harvest, six plants were taken at random. At the first four harvests, gas exchange, chlorophyll fluorescence, water relations, ion contents, and chlorophyll contents were measured on leaf 3. At the last two harvests only ion and chlorophyll contents were measured.

3.2.2 Water relations measurements

Tissue water potential (ψ) and osmotic potential (ψ_π) were measured with leaf-cutter psychrometers (J.R.D. Merrill Specialty Equipment, Logan, UT, USA). Leaf disks were sampled from around the mid-portion of leaf 3 using a 5-mm-diameter biopsy punch, and the three disks were transferred into a psychrometer chamber (Turner et al., 2000). After sampling, the psychrometers were equilibrated at 20°C for 3 h and then the total ψ was measured with a HP-115 water potential data system (Wescor, Logan UT, USA) operated in the psychrometric mode. Tissue osmotic potential (ψ_π) was measured on the same samples after freezing the tissue (still enclosed in the psychrometer chamber) in liquid nitrogen and then re-equilibrating the psychrometers at 20°C for 4 h. The psychrometers were calibrated using NaCl solutions, and the psychrometer chambers were carefully cleaned and dried as per the manufacturer's instructions after each use. Turgor was calculated as the difference of ψ_π from ψ.

3.2.3 Gas exchange measurements

Measurements of the rate of CO_2 assimilation and transpiration were made on the mid-portion of leaf 3 in the growth cabinet using a LI-6400 portable gas exchange system (LI-COR, Lincoln NE, USA). All measurements were taken between 4 to 5 h into a 9 h photoperiod and settings were chosen to match the growth cabinet conditions. Leaf temperature was maintained at 25°C, light intensity was set at 1150 μmol m^{-2} s^{-1} with a red/blue light source, CO_2 was set at 400 μbar and the leaf to air vapour pressure deficit kept between 1.0 to 1.5 KPa.

3.2.4 Chlorophyll fluorescence

All fluorescence measurements were made on intact leaves using a PAM-2000 chlorophyll fluorometer (Walz, Effeltrich, Germany) on the mid portion of leaf 3.

Maximal fluorescence yield (F_m) and minimal fluorescence yield (F_o) were measured and the end of the 14.5 h dark period. Variable fluorescence (F_v) was calculated from F_m and F_o (F_m-F_o) and the maximum quantum yield of photosystem II (PSII) was calculated as the ratio of F_v:F_m. Steady state light fluorescence measurements were taken 4 h into the light period (equivalent to midday) on the same day as the dark fluorescence measurements. Maximal fluorescence in the light (F_m') was measured after a pulse of saturating light, with steady state yield of fluorescence in the light (F_t) being measured immediately prior to this pulse. The quantum yield of PSII (ϕ_{PSII}) was determined as $\phi_{PSII} = (F_m' - F_t)/ F_m'$ (Genty et al., 1989). Minimal fluorescence yield in the light (F_o') was determined by covering the leaf with a darkening cloth and applying a far red light after switching off the actinic light. Photochemical quenching coefficient (qP) was calculated using F_o', according to the following equation: $qP = (F_m' - F_t)/(F_m' - F_o')$ and non-photochemical (NPQ) as: $NPQ = (F_m - F_m')/ F_m'$.

3.2.5 Leaf chlorophyll measurements

Leaf chlorophyll content was estimated using a hand-held SPAD 502 meter (Minolta, Osaka, Japan). Average SPAD chlorophyll readings were calculated from 5 measurements from the leaf tip to the leaf base. A linear relationship between SPAD chlorophyll estimates and total chlorophyll content was verified on plants grown in control conditions. Fresh leaf tissue was ground with a glass homogeniser in 2 ml 80% acetone (v/v), the homogenate was centrifuged at 5, 000 rpm for 2 min, and total chlorophyll content ($\mu g\ cm^{-2}$) was calculated from absorbances at 646.6 nm, 663.6 nm and 710 nm (Porra et al., 1989). Total chlorophyll determinations correlated well (r^2 = 0.94) to SPAD values measured on the same tissue before extraction (Figure 3.1). Importantly, there was a similar relationship between SPAD values and chlorophyll content estimation for both Wollaroi and Line 255 and therefore the SPAD chlorophyll meter was used to give an accurate rapid estimate of total leaf chlorophyll.

3.2.6 Ion analysis

The mid-portion of the blade of leaf 3 used for gas exchange and fluorescence measurements and the blades of main stem leaves 1, 2 and 4 were harvested separately, dried at 70°C for 3 days, weighed and extracted in 500 mM HNO_3 at 80°C for 1 h and analysed for Na^+ and K^+ by an atomic absorption spectrophotometer (Varian Spectra

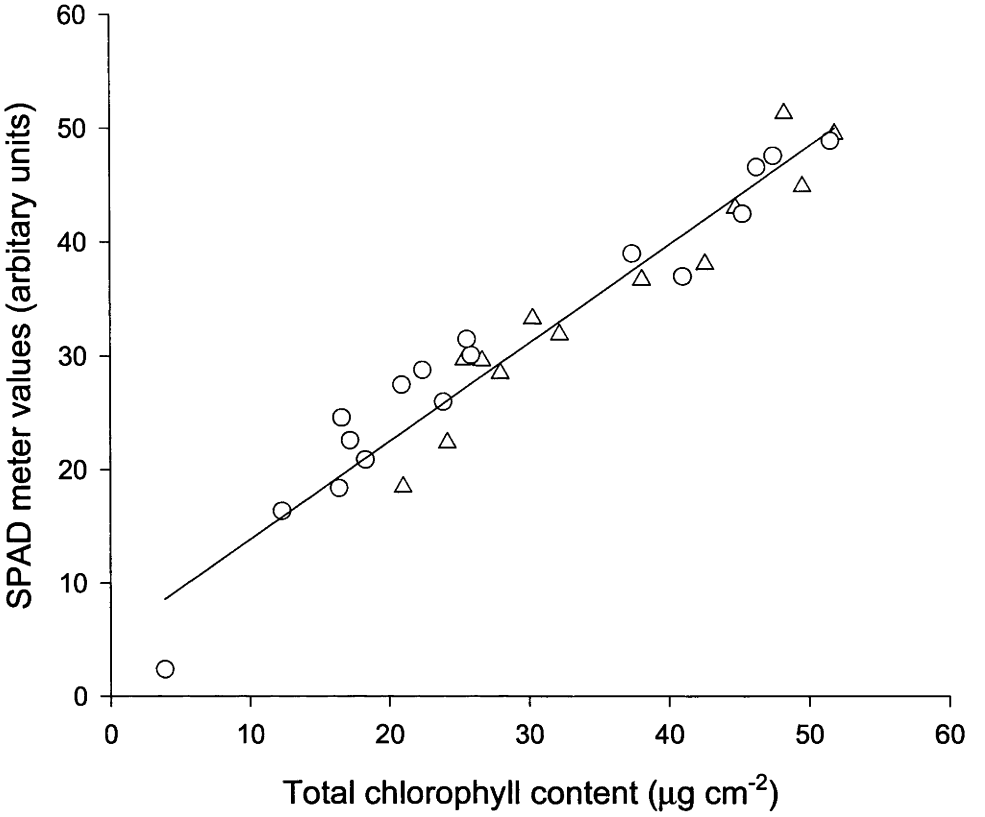

Figure 3.1 Relationship between leaf chlorophyll content and SPAD readings of Wollaroi (Δ) and Line 255 (O) in control conditions. Fitted linear regression is described by the following equation: $y = 0.86x + 5.24$ ($r^2 = 0.94$).

AA-300, Melbourne Australia). Chloride analysis was carried out using the same extracts with a specific ion (Cl^-) electrode (Model 96-17, Orion, Cambridge MASS, USA). Ions were determined in leaves from the salt treatment only. Na^+ and Cl^- concentrations in control treatments in other genotypes grown under similar conditions were very low (Rivelli et al., 2002).

3.3 RESULTS

3.3.1 Growth, leaf injury and ion accumulation

Shoot biomass accumulation of both genotypes was reduced when grown in 150 mM NaCl, but significant differences between Wollaroi and Line 255 were found only after 20 d (28 DAE). Shoot biomass of Line 255 was 5% higher than that of Wollaroi at 28 DAE, increasing to about 30% higher at 35 DAE (Figure 3.2).

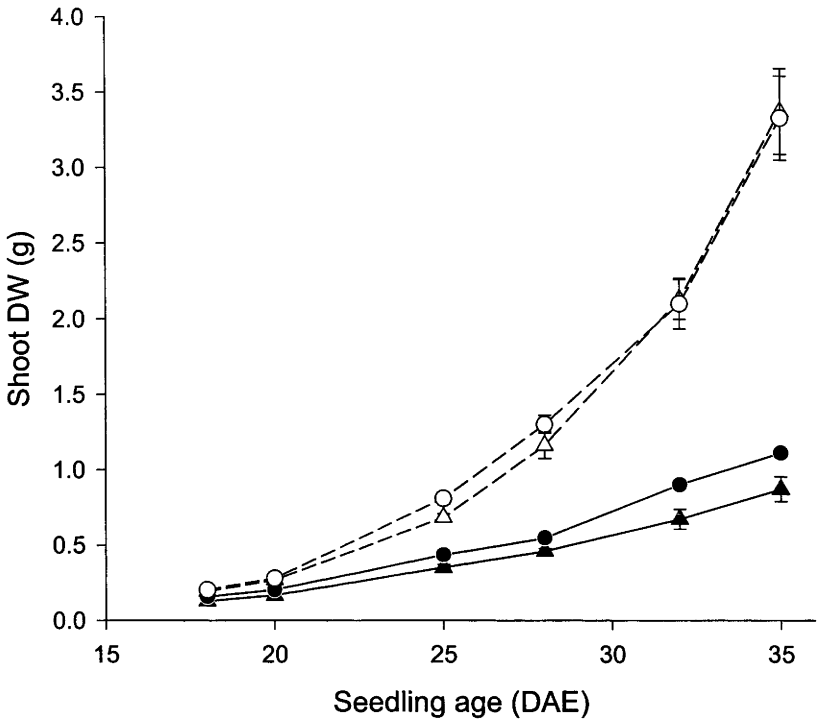

Figure 3.2 Effect of 150 mM NaCl on shoot dry weight of Wollaroi (Δ,▲) and Line 255 (○,●). Final salt addition was added at 8 DAE. Control data are represented by open symbols, and salt treatment by closed symbols. Values are means (n=6) ± s.e.

Genotypic differences in leaf injury occurred earlier than in shoot biomass and were noticeable after 12 d (20 DAE) of salt. At this time, the chlorophyll content of leaf 1 of Line 255 had decreased to 30% of controls, but leaf 1 of Wollaroi was dead (Table 3.1). The genotypic differences in injury continued with time, and at 35 DAE, leaves 3 and 4 (on the main stem) of Line 255 had significantly higher levels of chlorophyll than the equivalent leaves of Wollaroi (Table 3.1).

No leaves on control plants showed any signs of death or chlorosis. When the leaves were young, there was no difference in Wollaroi in the chlorophyll concentration of control and salt-treated plants, but in Line 255, salt treatment caused a greater increase in chlorophyll concentration per unit leaf area, than in controls. This is shown for leaf 3 in Table 3.1 at 20 DAE. Leaf width and length was reduced by salinity, so the higher chlorophyll concentration was presumably due to smaller cells and a higher chloroplast concentration per unit area. Figure 3.3A shows a negative relationship between chlorophyll content and Na^+ content of leaves 1 to 4 of Wollaroi and Line 255, sampled at each harvest throughout the duration of the salt treatment. Chlorophyll content in Line 255 was always higher

Table 3.1 Leaf injury assessed using an estimation of chlorophyll content of leaves 1 to 4 of Wollaroi and Line 255, from control and salt treatment (150mM NaCl) at 20 DAE and 35 DAE (seedling age). Values are averages (n=6) ± s.e.

	Chlorophyll content (SPAD units)			
	Wollaroi		Line 255	
	Control	Salt	Control	Salt
20 DAE (12 d in salt)				
Leaf 1	36 ± 1	0	36 ± 1	11 ± 4
Leaf 2	36 ± 1	27 ± 2	33 ± 1	31 ± 1
Leaf 3	38 ± 1	39 ± 2	38 ± 1	43 ± 1
35 DAE (27 d in salt)				
Leaf 1	35 ± 3	0	23 ± 4	0
Leaf 2	28 ± 2	0	26 ± 3	0
Leaf 3	36 ± 1	0	35 ± 1	14 ± 5
Leaf 4	35 ± 1	13 ± 4	30 ± 2	37 ± 3

than in Wollaroi for a given Na^+ concentration measured in the same leaf, even though chlorophyll content in the control condition was the same (Table 3.1). Consistent with this association, it appears that Line 255 has a greater capacity than Wollaroi to accumulate Na^+ in a leaf before its eventual death. Na^+ content at zero chlorophyll in Line 255 was above 4000 μmol g DW^{-1} compared to 2500 – 3000 in Wollaroi (Figure 3.3A). This trend appears to be similar for each of the main stem leaves that were monitored. There also appears to be a negative relationship between Cl^- accumulation and chlorophyll degradation, although it is less clear whether there were any differences between the two genotypes (Figure 3.3B).

3.3.2 Control of ion accumulation

Both genotypes exhibited good control of Na^+ accumulation in the blade of leaf 3 over the first half of the measurement period, indicated by a 'lag phase' between 7 and 15 d (Figure 3.4A). This control was more evident in Line 255, which also had slightly lower Na^+ concentrations than Wollaroi between 10 and 20 d.

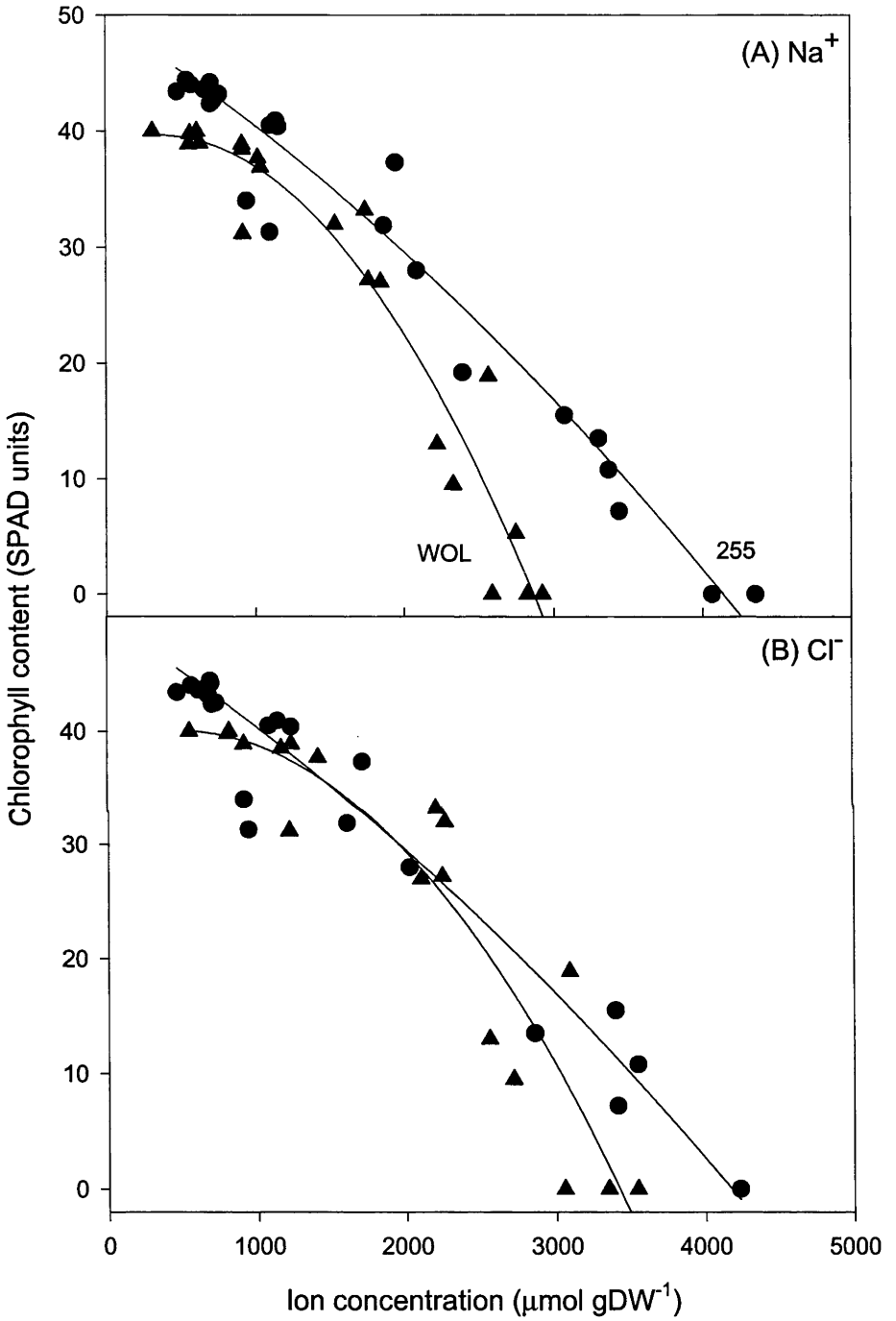

Figure 3.3 Relationship between the concentration (µmol gDW^{-1}) of (A) Na^+ and (B) Cl^- and chlorophyll content (estimate using SPAD meter) of main stem leaves 1 to 4 from Wollaroi (▲) and Line 255 (●) grown in 150 mM NaCl. Fitted curves are described by the following 2nd order polynomial regressions: Wollaroi (Na^+): $y = -6E\text{-}06x^2 + 0.0044x + 38.9$ ($r^2 = 0.93$); Line 255 (Na^+): $y = -9E\text{-}07x^2 - .0080x + 49.5$ ($r^2 = 0.98$); Wollaroi (Cl^-): $y = -5E\text{-}06x^2 + 0.0044x + 39.0$ ($r^2 = 0.88$); Line 255 (Cl^-): $y = -8E\text{-}07x^2 - .0084x + 49.6$ ($r^2 = 0.97$); Each value represents an average of 6 leaves.

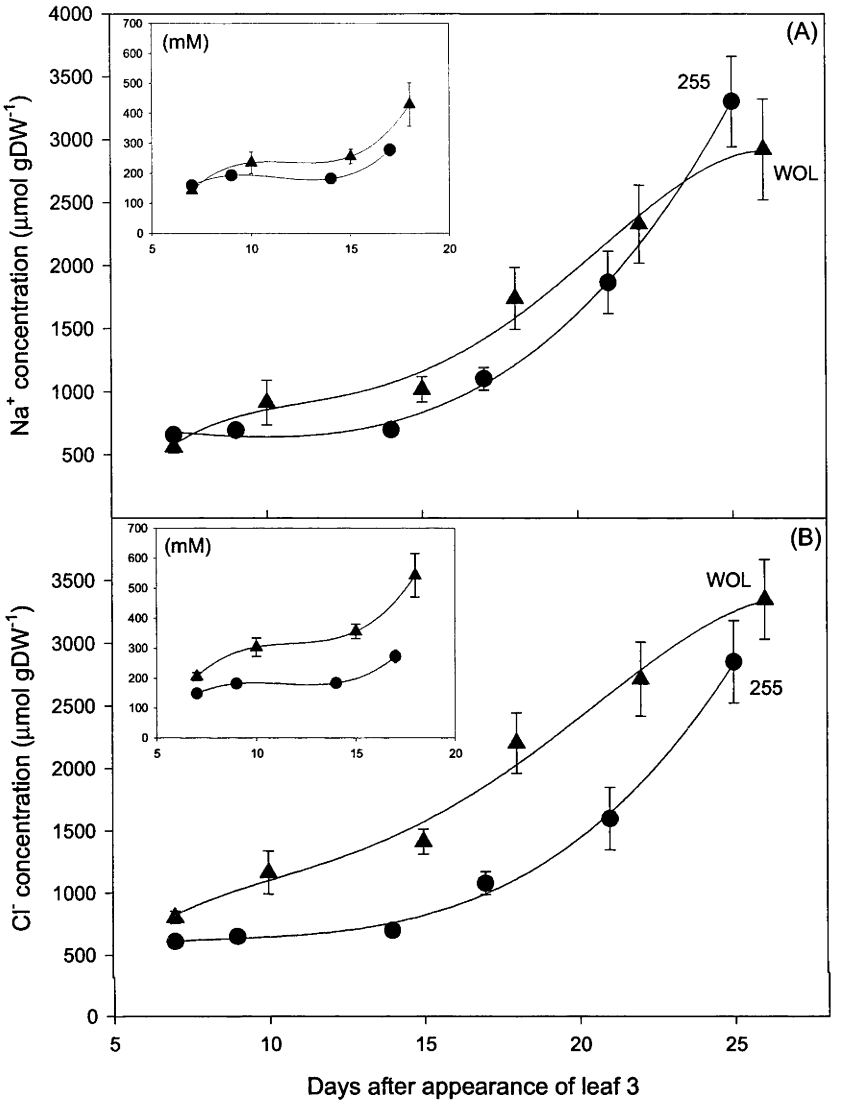

Figure 3.4 Concentration (µmol gDW^{-1}) of (A) Na^+ and (B) Cl^- in leaf 3 of Wollaroi (▲) and Line 255 (●) grown in 150 mM NaCl for 26 days. NaCl treatment commenced prior to the appearance of leaf 3. Inserts show the same data for the first four harvests calculated as a concentration on a tissue water basis (mM). Fitted curves are derived from 3rd or 4th order polynomial regressions. Values are averages (n=6) ± s.e.

Similarly, there appeared to be tight control of Cl^- accumulation in the blade of Line 255 between 7 and 14 d, as there was no increase in Cl^- for this period (Figure 3.4B). However, Wollaroi continued to accumulate Cl^- to a much greater degree, being about twice as high as Line 255 between 10 to 20 d in salt. The concentrations of Na^+ and Cl^- in Wollaroi and Line 255 were also calculated on a tissue water basis (mM) and displayed as inserts in Figure 3.4. The concentrations of Na^+ and Cl^- in Line 255 stayed below 200 mM until the fourth harvest, when they were calculated as 270 mM. In contrast, Na^+ and Cl^- in Wollaroi rose to 430 and 540 mM respectively. Line 255 was

still accumulating Na^+ after 25 d, while Wollaroi had ceased doing so as the leaf by this time was dead (Figure 3.4A).

3.3.3 Water relations

The water relations of leaf 3 from control and salt-treated durum seedlings are summarised in Figure 3.5. The salinity treatment resulted in a decrease in leaf water potential (ψ) and osmotic potential (ψ_π) in both genotypes (Figure 3.5A, 3.5B); however, there was a much greater decrease of ψ and ψ_π in Wollaroi than Line 255, over the steady period of the osmotic stress (between 10 – 15 d). There was an initial increase in leaf turgor of about 0.2 MPa in salt treated leaves of both genotypes relative to the controls, largely due to a substantial decrease in ψ_π (Figure 3.5C, 3.5B). By 15 d, the differences in turgor had increased to about 0.4 MPa. While the balance between ψ and ψ_π was maintained in salt-treated Line 255, leading to the same turgor over the duration of the experiment, the turgor of salt-treated Wollaroi decreased between 15 and 18 d due a greater decrease in ψ than ψ_π of about 0.3 MPa.

The contribution of the major ions (Na^+, K^+ and Cl^-) to ψ_π is summarised in Table 3.2. The sum of Na^+, K^+ and Cl^- in both genotypes contributed substantially to ψ_π, the contribution increasing to very high proportions with time. This may have been due to the translocation of organic solutes from the senescing leaf as Na^+, K^+ and Cl^- did not increase. Na^+ and Cl^- were responsible for the major part (70 - 90 %) of the ion component of ψ_π, with Cl^- alone in Wollaroi responsible for about 50% of the major

Table 3.2 Osmotic potential (ψ_π) and the contribution of Na^+, K^+, and Cl^- to the ψ_π in leaf 3 of Wollaroi and Line 255 grown in 150 mM NaCl. Values are means (n=6) ± s.e.

Genotype	Leaf 3 age (d)	$Na^+ + K^+ + Cl^-$ (MPa)	Ψ_π (MPa)	Ion component of Ψ_π (%)
Line 255	9	-1.35 ± 0.03	-1.72 ± 0.09	78
	17	-1.66 ± 0.09	-1.83 ± 0.19	91
Wollaroi	10	-1.82 ± 0.13	-2.10 ± 0.26	87
	18	-2.70 ± 0.29	-2.78 ± 0.30	97

ions (data not shown). Total ions in Wollaroi accounted for a greater component of ψ_π than in Line 255 at both the early period of osmotic stress (9, 10 d) and at the end of the measurement period (17, 18 d).

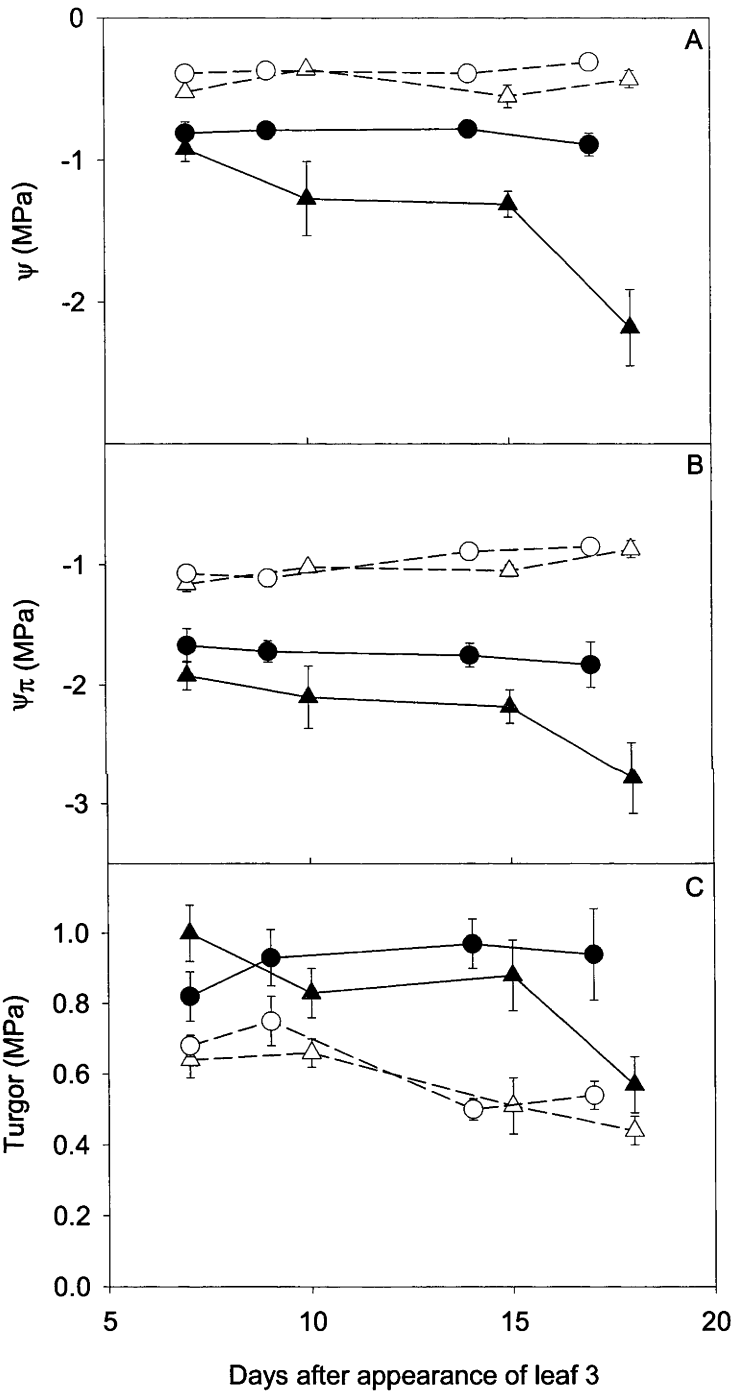

Figure 3.5 Effect of 150 mM NaCl on the (A) leaf water potential - ψ, (B) osmotic potential - ψ_π and (C) turgor of leaf 3 of Wollaroi (Δ,▲) and Line 255 (○,●) between 7 to 18 d. NaCl treatment commenced prior to the appearance of leaf 3. Control data are represented by open symbols, and salt treatment by closed symbols. Values are averages (n=6) ± s.e.

3.3.4 Effect of salt treatment on stomatal conductance and CO_2 assimilation rate

Stomatal conductance (g_s) of both genotypes in the salt treatment was already 40 to 50% less than that of control plants when gas exchange measurements commenced and

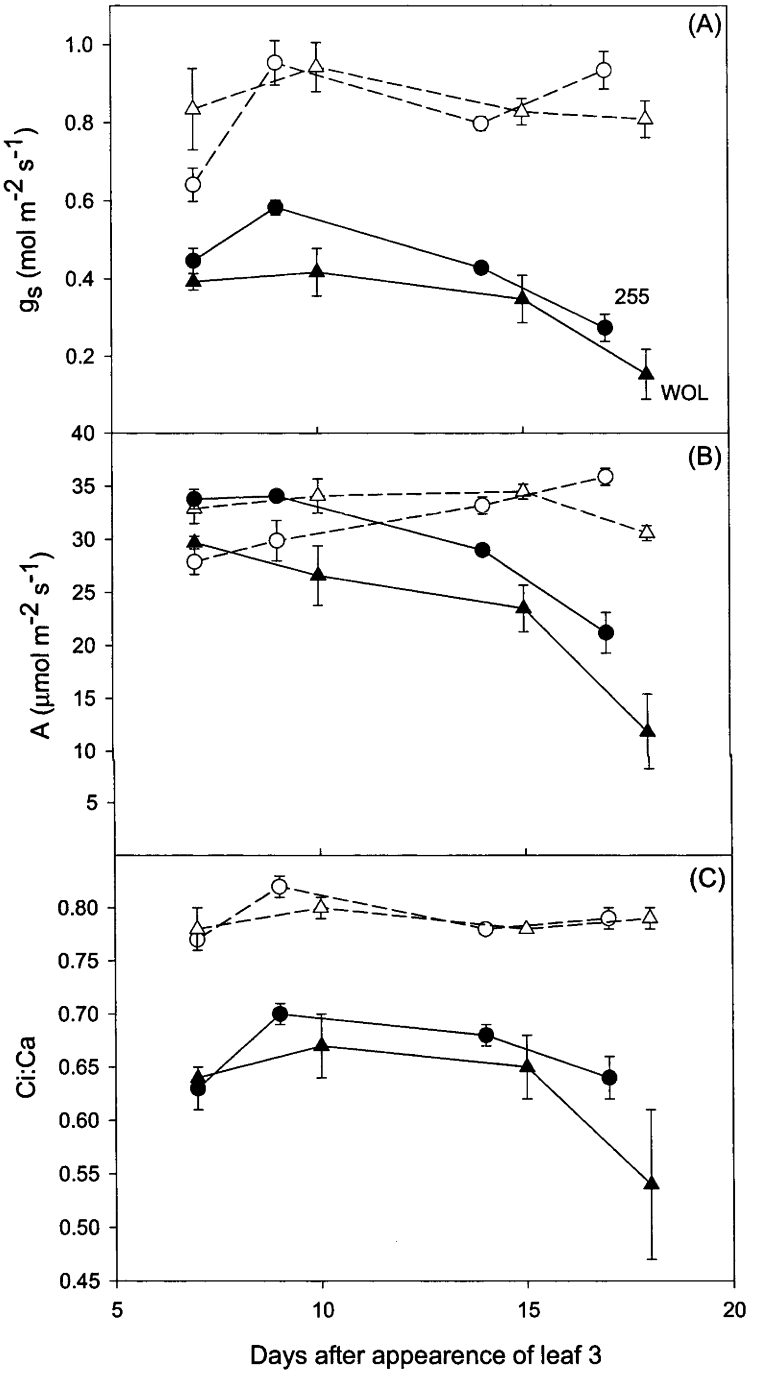

Figure 3.6 Effect of 150 mM NaCl on A) stomatal conductance (g_s – mol m^{-2} s^{-1}), B) CO_2 assimilation rate (A – µmol m^{-2} s^{-1}) and (C) Ci:Ca of leaf 3 of Wollaroi (Δ,▲) and Line 255 (○,●) between 7 to 18 d. NaCl treatment commenced prior to the appearance of leaf 3. Control data are represented by open symbols, and salt treatment by closed symbols. Values are averages (n=6) ± s.e.

decreased further to about 20% of the controls (Figure 3.6A). However, the rate of CO_2 assimilation (A) was largely unaffected at first, but then decreased with time (Figure 3.6B). The Ci:Ca was initially about 20% lower than the controls, was steady through most of the time and decreased at the end of the experimental period (Figure 3.6C). These data indicate that there was increased photosynthetic capacity of salt-stressed Line 255 seedlings compared to controls, as there was little or no change in A associated with a reduction in both Ci:Ca and g_s. Figure 3.7 summarises the relationship between Ci:Ca and A of both genotypes. The arrows on this figure indicate the change in Ci:Ca and A for the salt-treated plants that was not associated with stomatal closure, from the first to the second harvest. The remaining data from salt-treated plants fall on the same curve. The shape of the curve indicates that there were both stomatal and non-stomatal limitations associated with a decline in the A for both genotypes.

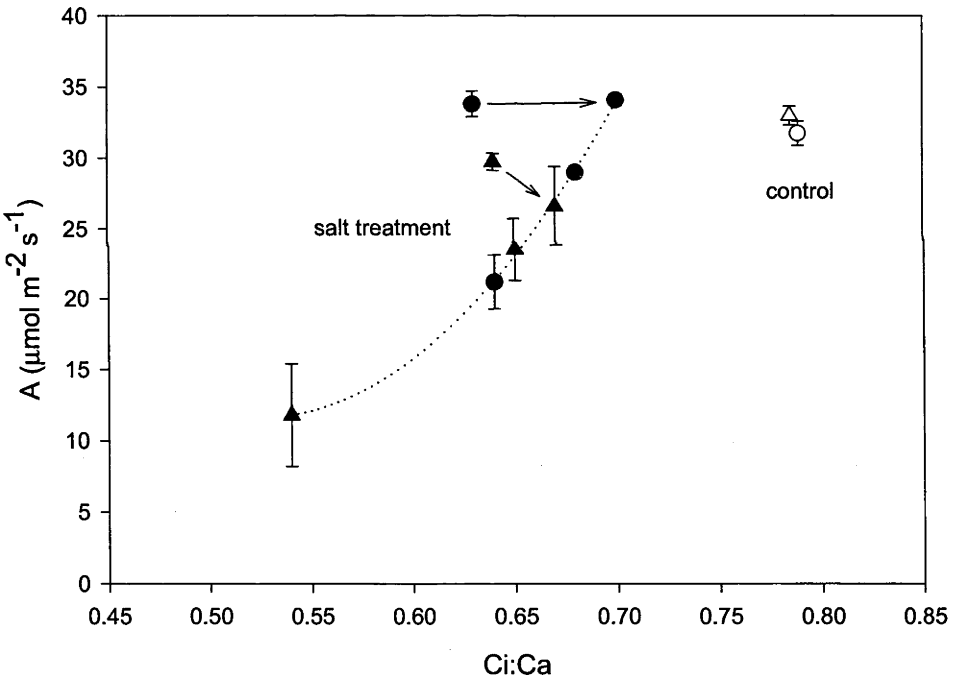

Figure 3.7 Relationship between CO_2 assimilation rate (A) and Ci:Ca in leaf 3 of Wollaroi (▲) and Line 255 (●) grown in 150 mM NaCl for 18 d. Arrows indicate a change in Ci:Ca from harvest 1 to harvest 2, the dotted line then indicating the trend of declining A with Ci:Ca for subsequent harvests in the salt treatment. Salt treatment values are averages (n=6) ± s.e. Open symbols represent the control data for Wollaroi (Δ) and Line 255 (O) (averages ± s.e. of 24 leaves from first 4 harvests).

Genotypic differences in g_s and A were less obvious than treatment effects. For salt-treated plants, g_s was similar for the two genotypes for most of the experimental period,

but A was consistently higher in Line 255 compared to Wollaroi (Figure 3.6). In Line 255, A only began to decline below that of the controls between 9 and 14 d, while A from salt-treated Wollaroi was already 10% below the control at 7 d and had fallen by a further 10% by 10 d. The value of A in salt-treated Line 255, was higher than controls between 7 – 9 d and control values of A for both genotypes remained relatively constant throughout the treatment period.

Figure 3.8 shows the relationship between chlorophyll content and A for both genotypes. In the salt treatment, chlorophyll and A decrease concurrently with time. A 20% decrease in A of Wollaroi (between 7 and 15 d where g_s was relatively stable – Figure 3.6) was associated with only a 3% decrease in chlorophyll content. Similarly, a 15% decrease in A of Line 255 was associated with only a 3% decrease in chlorophyll content.

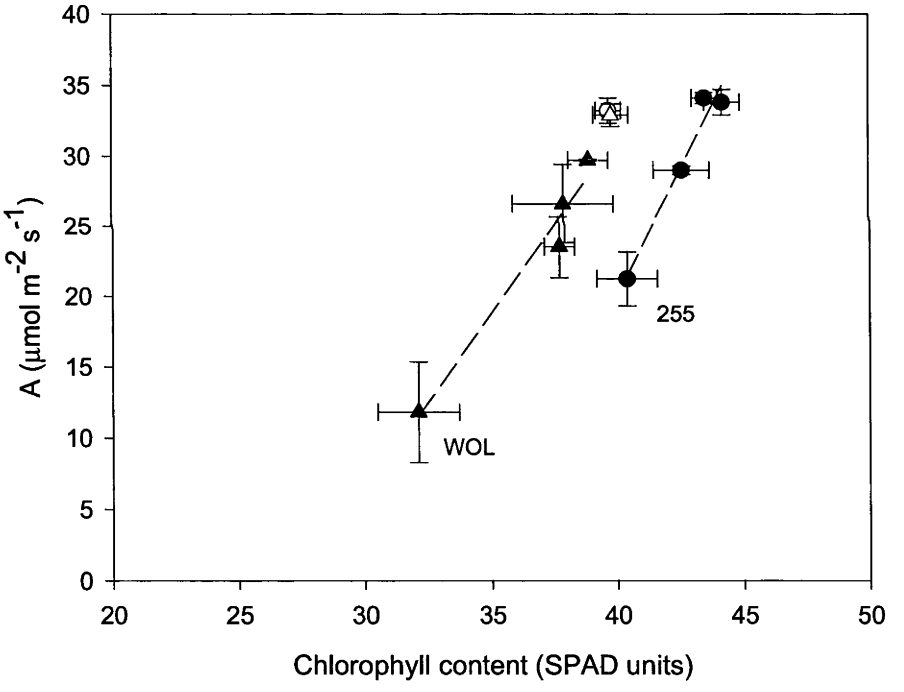

Figure 3.8 Relationship between chlorophyll content and CO_2 assimilation rate (A) in leaf 3 of Wollaroi (▲) and Line 255 (●) grown in 150 mM NaCl. Each data point represents an average of 6 plants (± standard errors) taken from the first four harvests between 7 to 18 d in salt treatment. Open symbols represent the control data for Wollaroi (Δ) and Line 255 (O) (averages ± s.e. of 18 leaves from first 3 harvests).

3.3.5 Effect of salt stress on chlorophyll fluorescence in durum wheat

Salinity had little effect on four key fluorescence parameters (Figure 3.9). The F_v:F_m of Line 255 remained unchanged for the 17 d in salt (Figure 3.9A). This is consistent with

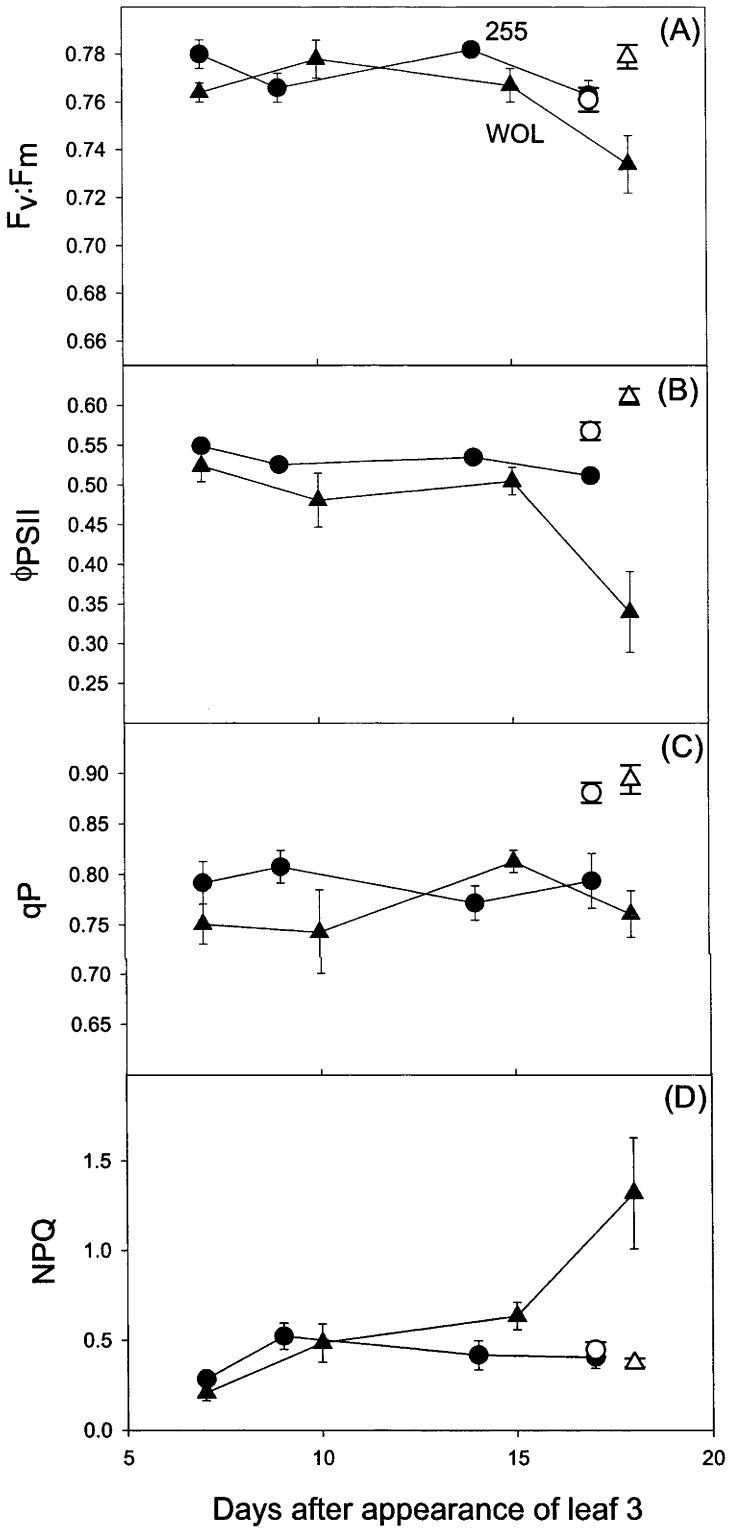

Figure 3.9 Effect of 150 mM NaCl on chlorophyll fluorescence parameters; (A) F_v:F_m (B) ϕPSII, (C) qP and (D) NPQ, on the intact leaf 3 of Wollaroi (Δ,▲) and Line 255 (O,●). NaCl treatment commenced prior to the appearance of leaf 3. Control data are represented by open symbols (values are averages ± s.e. of 24 plants from first 4 harvests), and salt treatment by closed symbols (values are averages (n=6) ± s.e. for each harvest).

other measured fluorescence parameters for Line 255, which also were unchanged over the course of the experiment (Figure 3.9B-D), signifying that there were no salt-induced decreases in the intrinsic or actual quantum efficiency of PSII, nor any associated increase in thermal energy dissipation. In contrast, Wollaroi showed a small but significant decline in $F_v:F_m$ after 18 days in salt, and concomitantly, a significant decline in the quantum yield PSII photochemistry (ϕ_{PSII}) at the end of this period (Figure 3.9C). While the proportion of PSII reaction centres that remained open (qP) was unchanged for both lines (Figure 3.9C), the portion of fluorescence quenching associated with thermal energy dissipation (NPQ) increased significantly only in Wollaroi between 10 and 18 d in the salt treatment (Figure 3.9D). In the control treatment, there was no decline in any of these parameters over the experimental period.

$F_v:F_m$ values of 0.76 – 0.78 in both control and salt treatment (Figure 3.6A) are lower than values considered optimal (0.83 – 0.85) for unstressed plants (Björkman and Demmig, 1987). This may be due to irreversible photoinhibition resulting from a sustained high PPFD over the course of the experiment (Bilger et al., 1995). Light intensity (direct and reflected) and duration of photoperiod (approx.1800 μmol m^{-2} s^{-1} for 9 h) and the type of light in growth cabinets (compared to sunlight) were such where this may have been a distinct possibility (see Sharma and Hall, 1991).

3.4 DISCUSSION

3.4.1 Growth and leaf injury

The growth of both wheat genotypes was substantially reduced by salinity, but genotypic differences in growth only started to appear after three weeks (Figure 3.2). This delay in genotypic differences is consistent with a two phase response to salinity model (Munns, 1993). Due to lack of genetic variation in the response to water stress imposed by the salt, genotypic differences in salt tolerance are likely to show up late rather than early and are associated with ion uptake and excessive ion accumulation in the leaves (Munns et al., 1995). In support of this, Nicolas et al. (1993) found that significant differences in salt tolerance between a salt sensitive durum cultivar and a salt tolerant bread wheat cultivar appeared only after 40 days.

The genotypic differences in dry matter production are likely to be accounted for by differences in net carbon assimilation rate, due either to differences in photosynthesis per unit leaf area, or to differences in photosynthesising area. Leaf injury, assessed as

loss of chlorophyll, was much greater in Wollaroi than Line 255, to the extent that one extra leaf on the main stem of Wollaroi was dead at the end of the experiment (Table 3.1). If salts arriving in the leaf can no longer be sequestered in vacuoles, and build up in the cytoplasm and cell walls, death will quickly follow (Munns and Passioura, 1984; Flowers and Yeo, 1986). In this experiment, while both genotypes demonstrated similar high early Na^+ accumulation, Line 255 eventually accumulated more Na^+ than Wollaroi (Figure 3.3), sustained less leaf injury and accumulated more biomass after 3 weeks. It would appear therefore that Line 255 has a superior ability to compartmentalise Na^+ which prolongs leaf life and therefore may increase the potential for prolonged supply of assimilate to the growing regions compared to Wollaroi.

3.4.2 Rate of ion accumulation

Genotypic differences in the rate of ion accumulation in the leaf blade were also noted as the leaf developed. In Line 255, the concentrations of both Na^+ and Cl^- in the leaf blade were relatively unchanged over the first two weeks, at about 200 mM for Na^+ and a little less for Cl^- (Figure 3.4). Wollaroi exhibited less control in ion transport over the same period as Na^+ and Cl^- contents increased to 260 mM and 360 mM respectively. The steady levels of ions in Line 255 were surprising as these leaves were transpiring at high rates over this time (Figure 3.6A) and both Na^+ and Cl^- would presumably have been transported from roots to shoots in the transpiration stream at a constant rate. The mechanism of this control of ion accumulation in the leaf blade may be through the retention of ions in the sheath tissue. Preferential partitioning of Cl^- into sheaths has been observed in salt-stressed sorghum, maize, wheat and barley (Boursier et al., 1987; Huang and Van Steveninck, 1989). Sodium has also been found to accumulate to a greater extent in the sheath than the blade of salt-stressed barley (Huang and Van Steveninck, 1989). Similarly, the capacity to exclude Cl^- from the mesophyll in preference to the epidermis in the blade of barley at high salinities, has also been suggested as an important factor contributing to salt tolerance in that species (Leigh and Storey, 1993).

3.4.3 Gas exchange

3.4.3.1 Stomatal versus non-stomatal effects

CO_2 assimilation was initially limited by stomatal conductance, and after 10 d by a combination of stomatal and non-stomatal factors (Figures 3.6, 3.7). This was obvious

in Wollaroi, but masked by an initial increase in photosynthetic capacity in Line 255 (Table 3.1, Figure 3.8). The contribution of stomatal versus non-stomatal limitations on photosynthesis may change with different levels of salt stress. Brugnoli and Björkman (1992) found that limitations on photosynthesis in cotton grown in 26% sea water (equivalent to 140 mM NaCl) were all stomatal; however, when the cotton was grown in 55% sea water (equivalent to 300 mM NaCl) non-stomatal and stomatal limitations were equal. Similarly, Everard et al. (1994) concluded that stomatal factors dominated at intermediate salinities (100 mM NaCl) in celery, whereas non-stomatal factors prevailed at high salinities (300 mM NaCl). Our results are consistent with these studies, as we found a contribution of both non-stomatal and stomatal factors implicated in the decline of A at a salinity level (150 mM) which for tetraploid wheat is a high salinity.

3.4.3.2 Osmotic effects

Reductions in photosynthesis due to stomatal factors are probably caused by osmotic effects on g_s. Stomatal conductance was already low when the first measurement was made (Figure 3.6A) and ion concentrations were low. Further, for both genotypes, a decline in stomatal conductance from about 0.55 mol m^{-2} s^{-1} to 0.35 mol m^{-2} s^{-1} was observed without any change in either Na^+ or Cl^- concentrations in the leaf (Figures 3.10C, 3.10D).

This lack of association between stomatal conductance and ion concentration has also been shown in four wheat genotypes differing in intrinsic Na^+ exclusion capacity, where stomatal conductance declined with time, without any corresponding increase in leaf ion concentrations even in the high Na^+ genotypes (Rivelli et al., 2002). Equally, poor water relations are unlikely to be the cause, as turgor had increased due to the salt treatment (Figure 3.5). These data point to another controlling factor, possibly a root signal in response to salt stress, which prompts stomatal closure regardless of leaf water status or ion concentrations (Termaat et al., 1985). Evidence for such a root signal affecting transpiration has also been found in drought-stressed wheat seedlings (Gollan et al., 1986; Passioura, 1988).

The initial enhanced capacity of A in Line 255, can be accounted for by an increase in chlorophyll content per unit leaf area. The decrease in SLA may be due to the osmotic effect of the salt on growth by producing smaller and thicker leaves, which had the effect of concentrating nitrogen and chlorophyll into a smaller area compared to

control leaves (see Table 3.1 and Figure 3.8). Further, it is unlikely that the following loss of photosynthetic capacity can be attributed to chlorophyll degradation as we found reductions in assimilation of about 20% in the early harvests to be associated with no

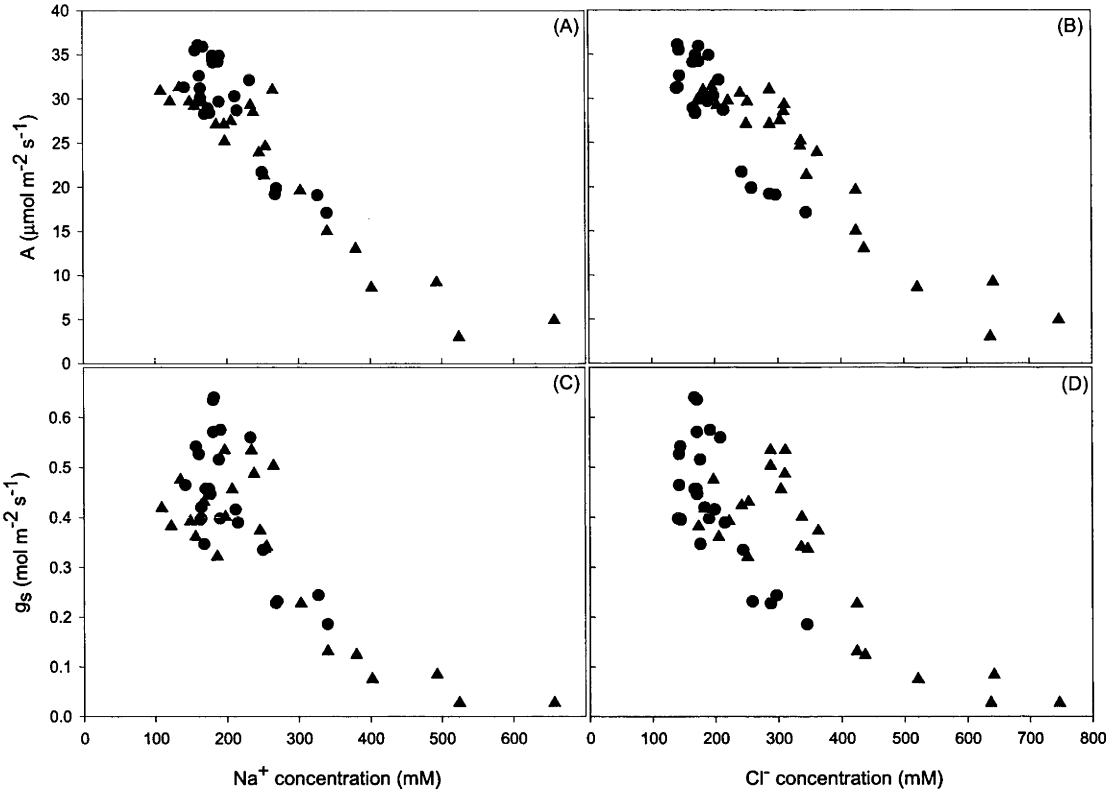

Figure 3.10 Relationship between gas exchange parameters and ion content (Na^+ and Cl^-) in leaf 3 of Wollaroi (▲) and Line 255 (●) grown in 150 mM NaCl. Each data point represents measurements from an individual leaf.

reductions in total chlorophyll content. Similarly, Yeo et al. (1985) found that photosynthesis in salt treated rice was reduced by 50% without any reduction in the concentration of chlorophyll. Additionally, Flowers et al. (1985) reported a decrease in photosynthesis in rice at salinity levels not affecting chloroplast ultrastructure, which they concluded was more sensitive to high cellular salt concentrations than was chlorophyll content.

3.4.3.3 Ion effects

Reductions in photosynthesis due to non-stomatal factors may be due to toxic ions. Correlations have been observed in a number of species including bean (Seemann and Critchley, 1985), cotton (Brugnoli and Björkman, 1992), citrus (Walker et al., 1982), grapevine (Downton, 1977) and rice (Yeo et al., 1985). Evidence in support of this

comes from strong negative correlations between ions and photosynthetic activity, where Na^+ has been implicated primarily in crop species such as rice (Yeo et al., 1985) and wheat (Rawson, 1986), and Cl^- in woody perennials such as citrus (Walker et al., 1993) and grape vines (Downton, 1977; Walker et al., 1981). Correlative data from the present experiment also show negative relationships between both Na^+ and Cl^- accumulation and CO_2 assimilation (Figures 3.10A, 3.10B). The concentration of Na^+ in the leaf blade corresponding to the start of a decline in A was about 200 mM and A was reduced a further 50% with Na^+ concentrations at about 350 mM. Chloride concentrations of about 300 mM corresponded to a start of a decline in A in Wollaroi, whereas there appeared to be no relationship between Cl^- and A in Line 255. Ion concentrations of this order can be detrimental to the integrity of the cell and affect photosynthetic processes directly through membrane damage or enzyme inhibition, if the vacuole can no longer sequester incoming ions. For example, Seemann and Critchley (1985) found that high Cl^- concentrations (250-300 mM) in the chloroplast of *Phaseolus* correlated with the efficiency of RuBP carboxylase. In that study, similar Cl^- concentrations were found in both the cytoplasm/chloroplasts and in the vacuole, indicating a breakdown in vacuolar compartmentation. Further experiments with genotypes differing in Na^+ and Cl^- accumulation are needed to fully resolve the contribution of these ions to the salt-induced decline in A

In contrast to these observations, different types of experiments have found poor correlations between ion accumulation and photosynthetic rates. For example, Tattini et al. (1995) observed a full recovery of CO_2 assimilation in olive relieved of a 200 mM NaCl stress, with leaf Na^+ contents remaining high during relief. Further, Rawson et al. (1988a) found different relationships between gas exchange and ion concentrations for different leaves and for different salinities.

3.4.4 Chlorophyll fluorescence

If high internal salt levels were to be toxic and directly affect the photosynthetic machinery then one might expect this to be reflected in the chlorophyll fluorescence measurements. No evidence of damage to PSII was found, as there were no changes in either the potential or actual quantum efficiency of PSII in Line 255, or in the earlier harvests of Wollaroi (see Figure 3.9). These results are therefore consistent with other studies, where F_v:F_m remained unchanged as chlorophyll concentration decreased in rice (Lutts et al., 1996), barley, (Morales et al., 1992), and sorghum (Sharma and Hall,

1992). Fluorescence data indicated that the decline in A in Wollaroi at the fourth harvest was a likely consequence of a direct toxic ion effect. At this time, while Na^+ and Cl^- increased to high concentrations in Wollaroi, ϕ_{PSII} and $F_v:F_m$ also declined (Figure 3.9). The proportion of PSII reaction centres that remained open (qP) was unchanged, indicating that this decrease in ϕ_{PSII} was unlikely to be due to feedback regulation caused by processes such as photoinhibition, but more likely from salt-induced photodamage. NPQ in Wollaroi increased noticeably during this period, indicating an increase in the thermal dissipation of excess light energy needed due to a salt-induced reduction in photosynthesis. However, it appears that this photoprotective process was not efficient enough as indicated by a substantial loss of chlorophyll for the same period (see Figure 3.8). There was a small reduction in qP in the last two measurements in both genotypes relative to their controls. This indicates the closure of a small proportion of PSII reaction centres, at least in light-adapted plants due to ongoing salt stress, and suggests a further down-regulation of PSII photochemistry, possibly matching reduced carbon acquisition due to the osmotic effects of salt stress (Delfine et al., 1998).

3.4.5 Prospects for screening durum wheat for tolerance to high internal salt concentrations

The results from this current experiment show the potential of screening for salt tolerance in durum wheat using symptoms of injury of leaf blades in conjunction with the measurement of salt ions in the same leaves. Genotypic differences in injury, quantified by measuring chlorophyll degradation in main stem leaves using a SPAD meter, showed up early after 12 d in salt treatment (20 DAE), were validated 2 weeks later (35 DAE) and predicted overall plant performance (Figure 3.2).

As noted earlier, CO_2 assimilation in the salt treatment was reduced before any significant decline in chlorophyll content, indicating that while chlorophyll content may be useful in predicting the long term effects of tissue tolerance capacity, it is insensitive to the immediate constraints from toxic ion concentrations. The effectiveness of chlorophyll fluorescence as a screening tool has been examined in a number of salt stress studies (Belkhodja et al., 1994; Lutts et al., 1996; Shabala et al., 1998), as a sensitive indicator of all factors that directly affect the photosynthetic apparatus. Observations from this current experiment indicate that most chlorophyll fluorescence parameters, particularly dark fluorescence measurements, are as equally insensitive to salt stress as chlorophyll itself and only reflect changes in photochemistry with

extremely high salt ion concentrations in the leaf. These results confirm similar findings from intact leaves of spinach (Delfine et al., 1999). However, in contrast to cotton grown at a higher salinity level (Brugnoli and Björkman, 1992), NPQ in Wollaroi began to increase after 10 d in salt, indicating a greater requirement to dissipate non-radiative energy, and thus probably reflected photochemical inefficiencies not yet detected in other light or dark fluorescence parameters. These results suggest that NPQ may be a useful parameter to indicate early non-visual symptoms of salt injury; however further experiments are required to validate this observation. From a practical viewpoint, measurements of chlorophyll content with the SPAD meter would be equally useful.

In conclusion, the study showed an association between increases in ion concentration and decreases in CO_2 assimilation rate. This was due partly to non-stomatal effects, suggestive of ion toxicity. However, these findings are only correlational. Further experiments using genotypes with greater contrasts in Na^+ and in Cl^- accumulation will be necessary to clearly separate osmotic from toxic ion effects.

Chapter 4

PHOTOSYNTHETIC CAPACITY IS RELATED TO THE CELLULAR AND SUBCELLULAR PARTITIONING OF NA^+, K^+ AND CL^- IN SALT-AFFECTED BARLEY AND DURUM WHEAT

4. PHOTOSYNTHETIC CAPACITY IS RELATED TO THE CELLULAR AND SUBCELLULAR PARTITIONING OF NA^+, K^+ AND CL^- IN SALT-AFFECTED BARLEY AND DURUM WHEAT

4.1 INTRODUCTION

The growth of wheat and barley plants in saline conditions is initially reduced because of a decrease in soil water potential. This is essentially a water stress resulting from the osmotic affect of salts in the soil solution. Growth can decrease further as a direct result of the salts taken up by the plant and accumulating in the older leaves to toxic concentrations. Once the capacity of the vacuoles to compartmentalise salt is reached, salts will build up in the cytoplasm, resulting in the disruption or impairment of important physiological and biochemical processes (Munns, 1993). Salt tolerance in a crop species such as wheat is usually associated with reduced uptake of Na^+ and maintenance of high K^+ in the shoots (Gorham et al., 1990b). Tolerance to high internal salt concentrations in the leaves is thought to be linked with the maintenance of low Na^+ concentrations in the apoplast (Oertli, 1968; Munns and Passioura, 1984; Mühling and Läuchli, 2002a), and low Na^+ and high K^+ concentrations in the cytoplasm (Leigh and Wyn Jones, 1984; Munns, 1993; Maathuis and Amtmann, 1999).

Low Na^+ and high K^+ in the cytoplasm are essential for the maintenance of a number of enzymatic processes (Munns et al., 1983; Bhandal and Malik, 1988) and protein synthesis (Flowers and Dalmond, 1992; Blaha et al., 2000); however, the cytoplasmic concentration at which Na^+ becomes toxic is not certain (Cheeseman, 1988). Munns (1993) suggested that non-toxic concentrations of Na^+ could possibly be as high as 100-150 mM. In contrast, Tester and Davenport (2003) concluded that maximum cytoplasmic Na^+ concentrations could be as low as 10 to 30 mM for plants grown in external salinities as high as 200 mM NaCl. The studies cited by Tester and Davenport (2003) all measured root cytoplasm concentrations and these may not be indicative of cytoplasm concentration in the leaf. Irrespective of what constitutes a toxic concentration, the avoidance of toxic concentrations of salt in the cytoplasm of mesophyll cells in the leaf depends on the effective sequestration of salt ions into leaf vacuoles. This in turn involves efficient and coordinated partitioning of ions at both the

cellular and sub-cellular levels. Patterns of cellular ion accumulation have been characterised in salt-affected barley leaves in a number of studies using a variety of analytical techniques, including X-ray microanalysis (eg. Huang and van Steveninck, 1989; Leigh and Storey, 1993), vacuolar sap extraction (eg. Fricke et al., 1996) and isolated protoplasts (eg. Dietz et al., 1992). Preferential partitioning of Cl^- and Ca^{2+} to epidermal cells was consistent in all these studies. A preferential distribution of Na^+ was less consistent, with higher concentrations in the epidermal cells at low salinities or under non-saline conditions (e.g. Karley et al., 2000a), but a more even distribution of Na^+ between epidermal and mesophyll cells at higher salinities. What is even less clear is the relationship between key physiological processes, such as photosynthesis, and the cellular compartmentation and (toxic) threshold cytoplasm concentrations of salt ions in salt-stressed plants. Only one study has attempted to link some of these parameters. Fricke et al. (1996) concluded that large increases in (vacuolar) mesophyll Na^+ concentration (to ~ 300 mM) were not associated with a relatively small reduction in photosynthesis (17%) of barley grown at 150 mM NaCl.

In Chapter 2 a large collection of tetraploid wheat accessions (*Triticum turgidum* L. ssp) was screeded, seeking genetic variation in symptoms consistent with tissue tolerance to high internal salt concentrations (Munns and James, 2003). Five tetraploid landraces were identified that maintained a high proportion of green leaves despite high leaf Na^+ contents. In Chapter 3 I investigated factors affecting CO_2 assimilation and leaf injury due to salinity in one of these tissue tolerant landraces, Line 255 and a salt-sensitive durum cultivar, Wollaroi (James et al., 2002). I found a negative relationship between CO_2 assimilation rate and leaf Na^+ and Cl^- concentration and concluded that Na^+ became potentially toxic at leaf concentrations of about 250 mM, which corresponded with the onset of non-stomatal reductions in photosynthesis.

In this chapter, the relationship between photosynthetic capacity and ion concentrations in the leaves of salt-stressed barley and durum wheat was further investigated with a particular emphasis on the cellular and subcellular partitioning of Na^+, K^+ and Cl^-. The concentrations of Na^+ and K^+ in the cytoplasm were also estimated and related to genotypic differences in cellular ion partitioning and photosynthetic capacity. Barley, a salt-tolerant species (Maas and Hoffman, 1977; Rawson et al., 1988b; Royo et al., 2000), possibly due to its ability to efficiently compartmentalise ions (Gorham et al., 1990a), was chosen as a tissue tolerant ideo-type and compared to a salt-sensitive Australian durum wheat cultivar – Wollaroi. In Chapter 2 Wollaroi had a

high degree of leaf injury associated with a relatively low leaf Na^+ concentration, when grown in 150 mM NaCl, indicating sensitivity to leaf Na^+ content. To select a barley variety with high tissue tolerance for comparison with Wollaroi, 11 different barley varieties were initially screened at a high salinity level and assessed for the relationship between percentage dead leaf and leaf Na^+ concentration.

The aims of this study were contingent on the ability to manipulate Na^+ content in order to examine photosynthetic capacity parameters over a broad range of tissue Na^+ concentrations but also to work in the time range before CO_2 assimilation rates naturally decrease with leaf age. Husain et al. (2004) found no further increase in shoot Na^+ concentration in wheat genotypes when grown in salinities above 50 mM NaCl. Additionally, Rivelli et al. (2002) found that there was no net increase in the leaf 3 Na^+ concentration of a similar collection of wheat genotypes between 10 to 30 d in 150 mM NaCl. For the study reported here, a number of salt treatments including a salt shock treatment (Storey and Wyn Jones, 1978) were therefore employed to break the tight control of Na^+ uptake into leaves and give a broad range of leaf Na^+ concentrations in leaf 3 between 2 to 14 days after full expansion.

4.2 MATERIALS AND METHODS

4.2.1 Growth conditions

Seeds were surface sterilised with 1% hypochlorite, and germinated in Petri dishes for 2 d. Germinated seeds were planted (one plant per pot) into 6.5 x 15.8 cm pots containing coarse quartz gravel, in a 90 L plastic moulded tray containing 153 pots in either glasshouse or growth cabinet with ambient conditions described below. Seedlings were watered using an automatic subirrigation system (Munns, et al., 1995; Munns and James, 2003), whereby solutions were pumped into trays and then drained into holding tanks every 30 min. For the salt treatment, seedlings were watered with half strength modified Hoagland's solution (P concentration reduced from 1 mM to 100 μM), then at 6 to 8 d after emergence (DAE), 25 mM NaCl was added twice daily until the desired final concentration was reached. Supplemental Ca^{2+} was also added as $CaCl_2$ to give a final Na^+: Ca^{2+} of 15:1, thereby avoiding Ca^{2+} deficiency. All solutions were changed fortnightly and pH was monitored and adjusted daily to between 6.0 – 6.5.

4.2.2 Experimental series

Experiment 1. Screening for barley tissue tolerance of Na^+

The following barley (*Hordeum vulgare* L.) cultivars were screened for tissue tolerance: Igri, Betzes, Maythorpe, Golden Promise, Clipper, Beecher, Skiff, O'Connor, Tantangara, Franklin and Schooner. Barley cultivars were grown in 150 mM NaCl in a glasshouse according to the method described above. Leaf 3 was harvested from seedlings after 10 d in salt treatment for Na^+ analysis. Subsequently, four randomly spaced replicate seedlings per barley cultivar were harvested at 35 DAE, which corresponded to 27 d in salt treatment. Leaf blades of these seedlings were separated into dead and green leaf portions and the percentage dead leaf was calculated from the dead leaf dry weight as a percentage of the total leaf dry weight. Average daily PAR was 31.8 mol m^{-2} day^{-1}. The average air temperature was 22.7°C (range: 20.4 – 24.6°C) during the day and 19.2°C (range: 17.4 – 21.0°C) during the night. Average root temperature was 19.7 °C (range: 18.6 – 21 °C) during the day and 19.6 °C (range: 18.8 – 20.4 °C) during the night.

Experiment 2. Manipulating Na^+ and Cl^- uptake by salt shock treatments

Durum cultivar Wollaroi (*Triticum turgidum* L. ssp. *durum* Desf.) and barley cultivar Franklin (*Hordeum vulgare* L.) were grown in a controlled environment chamber with a 10 h photoperiod and a maximum PPFD of 800 μmol m^{-2} s^{-1}, provided by 10 1000-W metal arc and 24 60-W incandescent lamps. The air temperature was controlled at 25°C during the day and 18°C during the night. Prior to the appearance of leaf 3 (6 DAE), three salt treatments commenced: 1) a high salinity treatment of 150 mM NaCl by incremental NaCl additions of 25 mM NaCl over 3 d, 2) a salt shock treatment where seedlings were subjected to 200 mM NaCl for 2 d before transferring to 150 mM NaCl, and 3) a salt shock treatment where seedlings were subjected to 250 mM NaCl for 2 d or 4 d before transferring to 150 mM NaCl via a transition step of 200 mM NaCl for 6 h. Leaf 3 was harvested after 10 d and 20 d from the commencement of the salt treatments and analysed for Na^+ and Cl^-.

Experiment 3. Photosynthetic capacity and the cellular distribution of Na^+, Cl^- and K^+ in leaf 3 of Wollaroi and Franklin grown in a range of salt treatments

Durum cultivar Wollaroi and barley cultivar Franklin were grown as in Experiment 2. Prior to the appearance of leaf 3 (7 DAE), three salt treatments commenced: 1) a low salinity treatment of 25 mM for 3 – 7 d before transferral to 150 mM NaCl by stepwise incremental NaCl additions of 25 mM over 2 d; 2) high salinity treatment of 150 mM NaCl by incremental NaCl additions of 25 mM over 3 d, and 3) a salt shock treatment where seedlings were subjected to 200 mM NaCl for 3 – 7 d before transferring to 150 mM NaCl. Gas exchange measurements were made on the mid portion of leaf 3, between 8 and 15 d old. Seedlings transferred from treatments 1 and 3 were left in 150 mM for at least 3 d to stabilize before gas exchange measurements were taken. The mid portion of leaf 3 used for gas exchange measurements was excised from the remaining leaf blade for Na^+, K^+ and Cl^- analysis. Leaf 3 blades from separate seedlings subjected to the same treatment were also sampled for cryo-SEM X-ray microanalysis.

4.2.3 Sampling leaf tissue for X-ray microanalysis

Two techniques were used to sample leaf sections for cryo-SEM X-ray microanalysis. Leaf portions were snap frozen using either a liquid ethane plunge or cryo-pliers. Ethane was kept in a frozen state in a brass crucible surrounded by liquid N_2 and liquefied (for about 2 mins) by carefully plunging a small brass rod into the frozen ethane. A 1 cm segment was cut from the mid part of leaf 3 and immediately plunged into liquid ethane. Frozen leaf segments were transferred from ethane into liquid N_2, placed into cryo-vials and stored in a liquid N_2 cryo-store. Flanking pieces of leaf tissue of similar size were also immediately sampled and placed into pre-weighed capped vials for accurate fresh weights. These samples were then dried at 70°C for 3 days, weighed for dry weight and calculation of water content and analysed for Na^+, K^+ and Cl^-.

The second freezing technique used pliers with adjustable brass plates in the jaws (described in McCully et al., 2000) which were cooled in liquid N_2 and then fastened onto the mid section of (an attached) leaf 3 for about 5 s. The cryo-pliers were then quickly placed into liquid N_2. The 2.8 cm leaf section was carefully removed from the brass face using a scalpel, transferred to a cryo-vial and stored in a liquid N_2 cryo-store. Flanking segments of leaf tissue were also sampled for Na^+, K^+ and Cl^- analysis, as above.

4.2.4 Cryo SEM and X-ray microanalysis

Frozen leaf segments were re-cut in liquid N_2 to expose an internal leaf surface for cryo-planing, and fixed to Al stubs using TBS Tissue Freezing Medium ™ (Triangle Biomedical Sciences, Durham, NC, USA). Stubs were transferred to a CR-X cryo-sectioning system (RMC Products, Boeckeler Instruments, Tucson AZ, USA) attached to a cryo-microtome (Ultracut E, Reichert-Jung, Vienna, Austria) and the leaf segments planed using glass and diamond knives at -90°C to a polished transverse face. Planed leaf samples were transferred in liquid N_2 to a cryo-transfer unit (Oxford Instruments, Eynsham, UK) attached to a cryo-SEM (JEOL 6400, JEOL Ltd., Tokyo, Japan), carefully etched at -89°C (to remove frost), cooled to between -170°C to -180°C, and coated with evaporated high purity aluminium. Images were recorded at 15 kV to a digital recorder (Imageslave, OED Pty Ltd., Hornsby, Australia). Contents of leaf cell vacuoles were analysed using the beryllium (Be) window with a Link eXL system (Oxford Instruments, Eynsham, UK).

Spectral data from the cryo-SEM for Na^+, Cl^- and K^+ were converted to elemental concentrations using 2nd order polynomial regressions derived from calibration curves of 10 – 500 mM standard solutions (Cheng Huang, unpublished data). The conversion equations for Na^+, Cl^- and K^+ were:

$$[Na^+]\ mM = 3.503 + (0.0881 \times NI_{Na}) + (0.00003 \times (NI_{Na})^2) \quad (1)$$

$$[Cl^-]\ mM = -0.454 + (0.0251 \times NI_{Cl}) + (0.0000004 \times (NI_{Cl})^2) \quad (2)$$

$$[K^+]\ mM = -2.775 + (0.0260 \times NI_K) + (0.0000002 \times (NI_K)^2) \quad (3)$$

where NI was the net peak integral of an element at a set live time of 150 s.

Standards were mixed with a graphite slurry to give 5% carbon in the mixture, and a few drops were snap frozen to Al stubs in liquid ethane. These standard solution samples were transferred to liquid N_2 and then prepared in an identical way to leaf samples for cryo-SEM and X-ray microanalysis. A more detailed description of this procedure is given by Huang et al. (1994) and McCully et al. (2000).

Where possible, mesophyll and epidermal cells were analysed throughout a cross section of the leaf blade to account for any variation in Na^+, K^+ and Cl^- concentration that may exist between the mid-vein and leaf margin. 6 – 15 spectra were collected for each cell type for each single leaf sample. Figure 4.1 displays a lightly

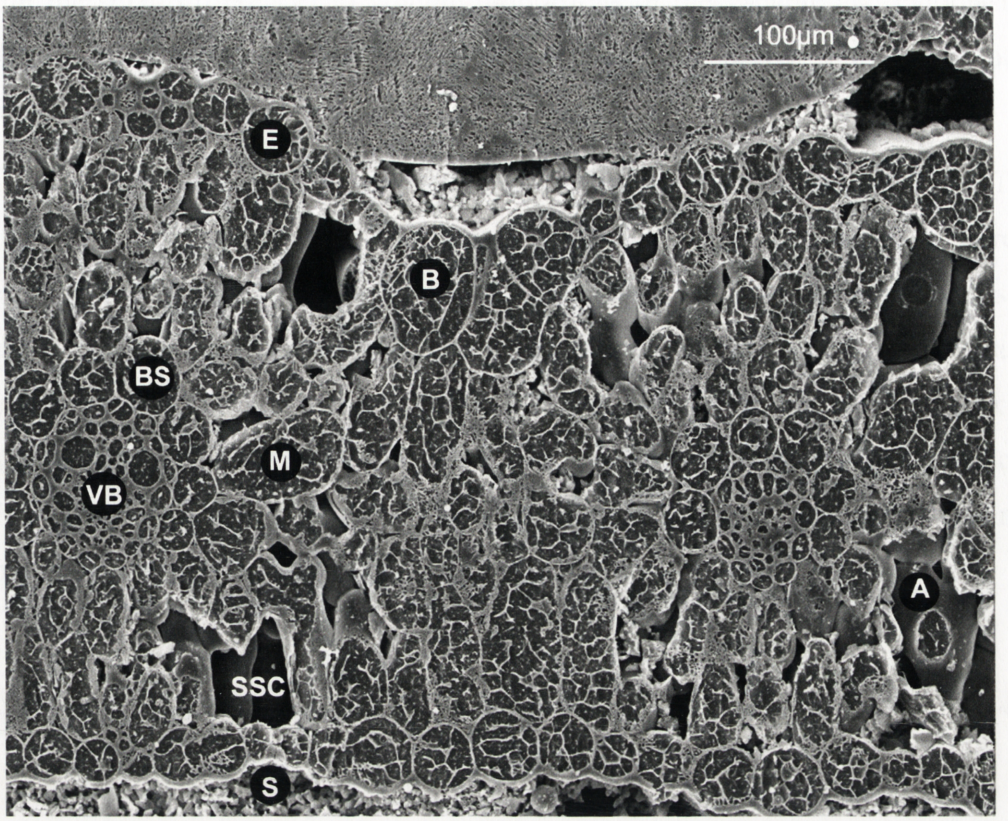

Figure 4.1 Labelled cryo-SEM micrograph of a snap frozen, cryo-planed and lightly etched transverse face of leaf 3 of salt-stressed durum wheat (cv. Wollaroi). VB= vascular bundle; BS= bundle sheath; M= mesophyll; E=epidermis; B=bulliform cell; S= stomate; SSC= sub-stomatal cavity; A= airspace; Bar = 100 µm.

etched cryo-SEM transverse face of a durum wheat leaf, typical of the preparations used at this stage of the study. Mesophyll cells were analysed in the areas between, above and below vascular bundles and epidermal cells were typically analysed from upper and lower surfaces in equal proportion to account for variation that exists between surfaces and location of epidermal cells (Fricke et al., 1994; 1995).

4.2.5 Estimation of Na^+ and K^+ concentration in the cytoplasm of mesophyll cells

To calculate the Na^+ and K^+ concentration in the cytoplasm, volume proportions of mesophyll, epidermal and vascular tissue and the subcellular compartments therein were taken from Winter et al. (1993), Harvey and Thorpe (1986) and Jellings and Leech (1984), as summarised in Table 4.1. As these previous studies were on barley or a range of wheat genotypes, the proportions were assumed to be similar for the barley and durum wheat leaves used in this present study. These calculations have also taken into

account the small differences in volume proportions of the vacuole and cytoplasm in mesophyll cells from salt-stressed leaves compared to leaves grown in control conditions (Harvey and Thorpe, 1986).

The total cytoplasm volume portion of the leaf was calculated to be 21% (Table 4.1). This was determined with the assumptions that Na^+ and K^+ concentrations were the same in the chloroplasts and cytosol and that cytosol in the mesophyll, epidermis and vascular tissue contained similar concentrations of these ions. The estimated vacuolar volume proportions are shown in Table 4.1. These values, together with the bulk leaf Na^+ (or K^+) concentration and vacuolar ion concentrations measured directly via cryo-SEM X-ray microanalysis, were used to estimate the Na^+ (or K^+) concentration in the cytoplasm as described in Equations 4 and 5:

$$Na^+_{leaf} = Na^+_{mes,vac} + Na^+_{epi,vac} + Na^+_{vein,vac} + Na^+_{cytoplasm} \quad (4)$$

$$[Na^+]_{cytoplasm} = \{[Na^+]_{leaf} - (0.402 \times [Na^+]_{mes,vac}) - (0.343 \times [Na^+]_{epi,vac}) - (0.045 \times [Na^+]_{vein,vac})\} \div 0.21 \quad (5)$$

where [ ] indicates concentration on a volume basis

Na^+ and K^+ were not directly measured in the vein tissue for each sample but estimated from cryo-SEM X-ray microanalysis which was performed on entire leaf veins (3 per genotype), avoiding the large mature xylem vessels.

The contribution of the apoplast was not included in these calculations. Winter et al. (1993) estimated that the apoplast and xylem together contributed only 5.9% to the aqueous volume proportion in barley leaves, indicating that the apoplast alone may not have contributed significantly to the aqueous volume of the leaf. Additionally, the relative contribution of the apoplast to leaf ion concentrations is assumed to be low. For example, apoplastic Na^+ remained below 10 mM in both salt-stressed wheat (Wimmer et al., 2003) and barley (Ramanjulu et al., 1999) grown in 75 mM and 100 mM NaCl respectively.

Table 4.1 Estimate of aqueous volume proportions of cell type and subcellular compartments in salt-stressed wheat and barley leaves, used for calculating cytoplasmic ion concentrations according to Equations (4) and (5).

Cell type	Volume proportion (of total leaf volume)	Vacuole (%)	Cytosol (%)	Chloroplast (%)	Cytoplasm[a] (%)	Volume proportion (of total leaf volume)	
						Vacuole	Cytoplasm
Mesophyll	0.60	67	9	24	33	0.402	0.198
Epidermis	0.35	98	2	-	2	0.343	0.007
Vascular	0.05	90	10[b]	-	10[b]	0.045	0.005
Total:	**1.00**					**79.0**	**0.210**

Estimates derived from Winter *et al.* (1993), Jellings and Leech (1984) and Harvey and Thorpe (1986).

[a]Cytoplasm is the sum of cytosol and chloroplasts.

[b]Value for vascular cells is an estimate of cytosol in the vein cells excluding xylem. The airspace fraction was estimated by these authors at 23 % (barley) and 25% (wheat).

4.2.6 Gas exchange measurements

Measurements of the rate of CO_2 assimilation (A) were made on leaf 3 from 18 to 23 d old seedlings in a constant environment cabinet using a LI-6400 portable gas exchange system (LI-COR, Lincoln NE, USA). All measurements were taken between 3 to 7 h into a 10 h photoperiod and settings were chosen to match the growth cabinet conditions. Leaf temperature was maintained at 25°C, light intensity was set at 800 µmol m^{-2} s^{-1} with a red/blue light source, ambient CO_2 was set at 400 µbar and the leaf to air vapour pressure deficit (VPD) maintained between 1.0 to 1.1 KPa with the daily addition of 4 ml dH_2O in the soda lime.

A:Ci response curves for individual leaves were obtained with a series of measurements, where A was initially measured after 10 – 15 min at ambient CO_2 (400 µmol mol^{-1}). To determine the initial slope of the A:Ci curve, the CO_2 concentration was gradually deceased to 50 µmol mol^{-1} (400, 300, 200, 100, 50). The CO_2 concentration was then returned to 400 µmol mol^{-1} before progressively increasing to 1400 µmol mol^{-1} (400, 500, 600, 800, 1000, 1200, 1400) to complete the curve. Photosynthetic capacity parameters, V_{cmax} and J_{max} were calculated by fitting the model of CO_2 assimilation proposed by Farquhar et al. (1980). V_{cmax} was determined from that part of an A:Ci curve where CO_2 is limiting, and is an estimate of maximum Rubisco

activity. J_{max} was calculated from that part of the curve where CO_2 is not limiting and represents the rate of RuBP regeneration, which is largely determined by the rate of electron transport (Farquhar et al., 1980; von Caemmerer and Farquhar, 1981).

4.2.7 Ion analysis

Leaf 3 blade segments were dried at 70°C for 3 days, weighed, extracted in 500 mM HNO_3 at 80°C for 1.5 h and analysed for Na^+ and K^+ by an atomic absorption spectrophotometer (SpectrAA-300, Varian, Melbourne, Australia). Chloride analysis was carried out using the same extracts with a specific ion (Cl^-) electrode (Model 96-17, Orion, Cambridge MASS, USA).

4.3 RESULTS

4.3.1 Barley screen for tissue tolerance of Na^+

A diverse range of 11 barley cultivars including winter and spring types, 2 row (malting) and 6 row (feed) varieties were grown in 150 mM NaCl for 27 d and screened to examine genotypic variation in (leaf) tissue tolerance to high Na^+ concentrations. The degree of leaf injury on the whole seedling was compared in relation to Na^+ concentration of leaf 3 after 10 d in salt, where the combination of a low degree of leaf death with high rates of leaf Na^+ accumulation would indicate greater tissue tolerance to high leaf Na^+ concentrations (Figure 4.2).

The accumulation of Na^+ in leaf 3 after 10 d varied from about 1100 (Beecher) to 1900 μmol gDW^{-1} (Franklin). These concentrations indicated reasonably high Na^+ uptake rates. The percentage dead leaf (% dead leaf) in the 11 barley cultivars ranged from about 4 to 15% after 27 d in salt (Figure 4.2). Six barley cultivars displayed a particularly low degree of leaf injury (4-5% dead leaf) indicating greater tissue tolerance, while the remaining cultivars appeared to be less tolerant, exhibiting higher degrees of leaf death of between 9 – 15%. From the cultivars tested in this study, Franklin was selected as the barley cultivar with the greatest tissue tolerance to high leaf Na^+ concentrations for comparison with the sensitive durum cultivar Wollaroi.

4.3.2 Manipulating short-term Na^+ and Cl^- uptake

In order to obtain a broad range of salt concentrations in leaf 3 between 2 to 14 days

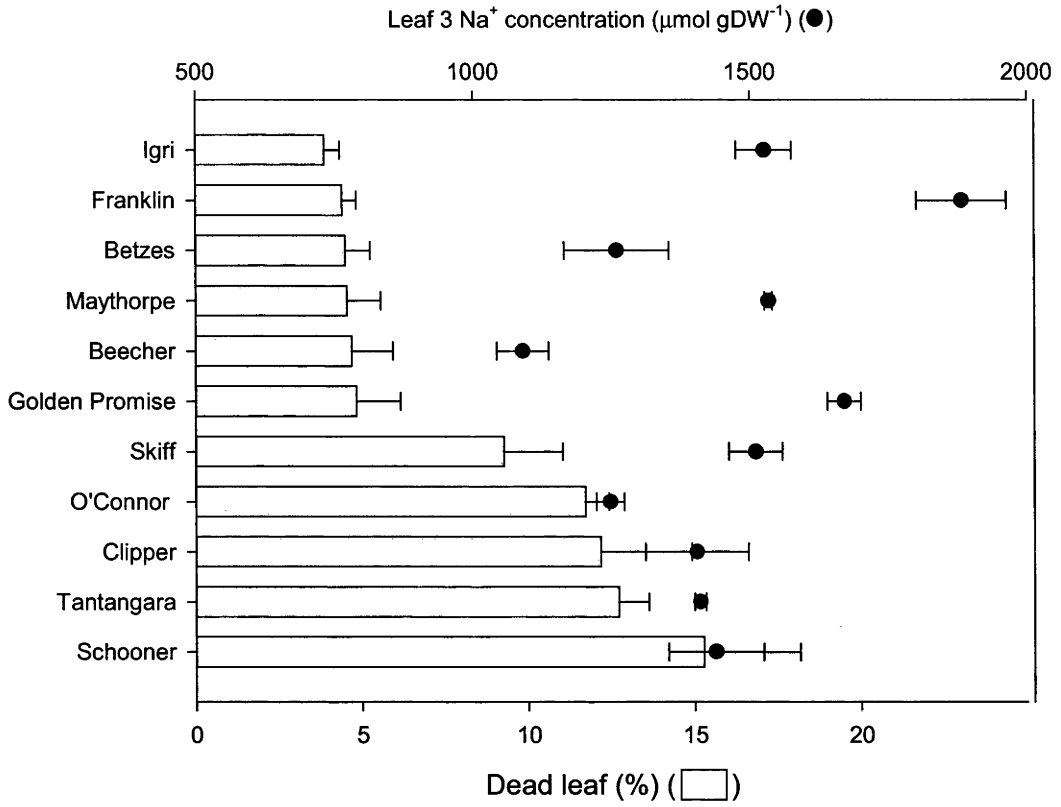

Figure 4.2 A screen of 11 barley cultivars for Na^+ concentration of leaf 3 (after 10 d in 150 mM NaCl) and percentage seedling dead leaf (after 27 d in 150 mM NaCl). Histogram bars represent average percentage dead leaf and data-points (●) represent average leaf 3 Na^+ concentration ± s.e. (n = 4).

after full expansion, a number of salt treatments including salt shock treatments were used. The effects of salt shock on Na^+ and Cl^- uptake and accumulation into leaf 3 were examined by comparing seedlings subjected to either a gradual incremental increase in NaCl to 150 mM or high-salt shock treatments of 200 mM and 250 mM NaCl imposed in a single dose.

The most effective treatment for raising the ion concentration in the leaves of the durum cultivar Wollaroi was gained from a 200 mM NaCl shock for 2 d (Table 4.2). This treatment doubled the average Na^+ and Cl^- concentration in leaf 3. The 250 mM NaCl shock treatment proved lethal for Wollaroi with few seedlings surviving beyond 10 d. Franklin barley demonstrated greater tolerance to the osmotic shock and the subsequent effects of increased ion concentrations in the leaves, with all seedlings surviving all salt shock treatments to 20 d. The 250 mM (4d) NaCl shock treatment proved most effective in raising leaf Na^+ and Cl^- concentrations in Franklin, with the Na^+ concentration increasing by about 40% and Cl^- concentration by 50%, compared to

Table 4.2 The effect of 'salt shock' on the accumulation of Na^+ and Cl^- in leaf 3 of Wollaroi and Franklin. Values are averages ± s.e. (n=10) after 10 d and 20 d in salt. Plants were returned to 150 mM NaCl after 'salt shock' treatment. Spaces indicate death of plants. Figures in brackets indicate the duration of the high-salt shock treatments.

Treatment	Wollaroi		Franklin	
	$[Na^+]$ (mM)	$[Cl^-]$ (mM)	$[Na^+]$ (mM)	$[Cl^-]$ (mM)
150 mM NaCl	200 ± 9	292 ± 18	283 ±12	309 ± 24
200 mM NaCl – shock (2d)	422 ± 96	566 ± 124	269 ± 16	350 ± 27
250 mM NaCl – shock (2d)	-	-	265 ± 14	345 ± 20
– shock (4d)	-	-	391 ± 37	455 ± 49

the 150 mM NaCl treatment. Leaf Na^+ concentrations in Franklin from the 200 mM (2d) and 250 mM (2d) NaCl shock treatments remained unchanged compared to the 150 mM treatment, whereas Cl^- concentrations increased by about 10%.

These salt treatments were therefore used to examine the relationship between ion concentration and photosynthetic capacity over a broad range of leaf salt concentrations and within a time range before CO_2 assimilation rates naturally decrease with leaf age.

4.3.3 Cellular and subcellular distribution of Na^+, K^+ and Cl^- in leaf 3 of salt-stressed barley and durum wheat

4.3.3.1 Distribution of Na^+, Cl^- and K^+ in the vacuole of mesophyll and epidermis cells

The concentration of Na^+ in the vacuoles of mesophyll and epidermal cells of leaf 3 from Franklin and Wollaroi seedlings grown in a range of salinities was measured using cryo-SEM X-ray microanalysis. These values were compared to bulk leaf Na^+ concentrations of flanking leaf segments calculated from ion and water contents of whole tissues (Figure 4.3).

A combination of 'salt shock' treatments, together with the sampling of plants grown in 25 mM and 150 mM NaCl resulted in a range in leaf Na^+ concentrations of 100 – 400 mM in the fully expanded 3rd leaf of Franklin and Wollaroi over a time period of between 9 – 18 d in salt.

Na^+ increased linearly in the vacuoles of mesophyll cells with increasing leaf Na^+ concentration (Figure 4.3). Na^+ in the vacuoles of epidermal cells also increased with increasing leaf Na^+ concentration although with greater variability, and was at lower concentrations than mesophyll cells in both genotypes at leaf Na^+ concentrations greater than 250 mM. Vacuolar Na^+ concentrations in Franklin were about 10% more in mesophyll cells than was present in the leaf as a whole (up to leaf concentrations of 400 mM), whereas, vacuolar Na^+ concentrations in Wollaroi were identical to the bulk leaf concentration in the range between 100 – 300 mM.

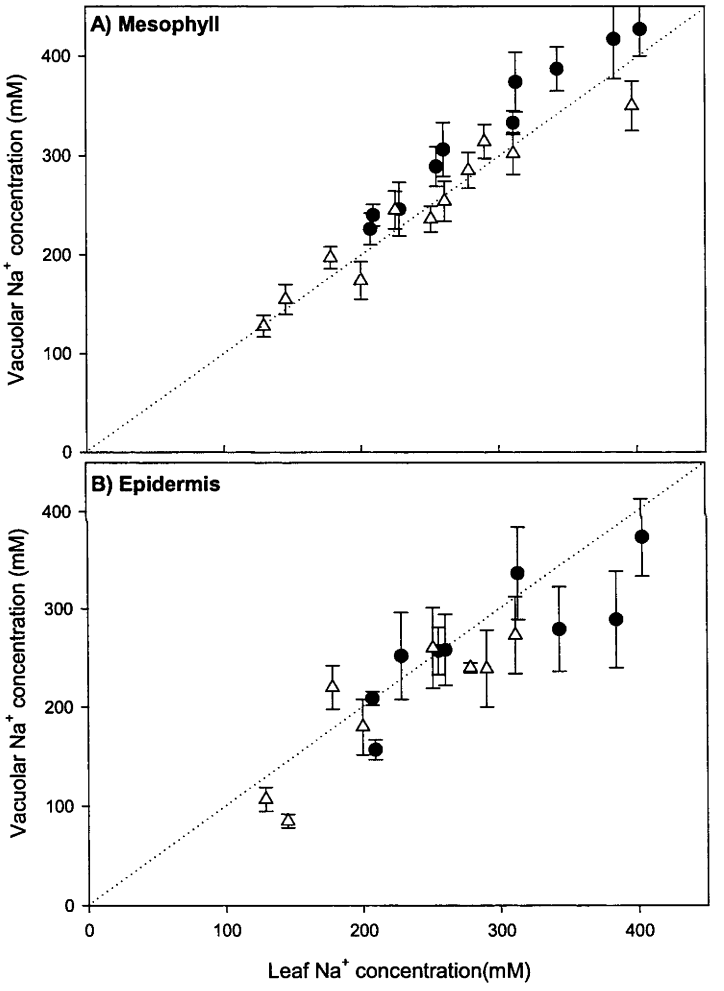

Figure 4.3 Relationship between bulk leaf Na^+ concentration and Na^+ concentration in the vacuoles of A) mesophyll and B) epidermal cells of leaf 3 of salt-stressed barley cultivar Franklin (●) and durum wheat cultivar Wollaroi (△). Vacuolar concentrations are given as means ± s.e. from 6 – 15 individual cells analysed by cryo-SEM X-ray microanalysis. Dotted line indicates 1:1 line.

Vacuolar K^+ concentrations in mesophyll and epidermal cells were linearly related to bulk leaf K^+ concentrations, with the exception of the mesophyll of Franklin,

where there was no discernable relationship between vacuolar K^+ concentrations and bulk leaf concentrations (Figure 4.4). K^+ increased to much higher concentrations in the mesophyll compared to the epidermis in both genotypes. Vacuolar K^+ concentrations were analysed by analysis of variance, and least significant differences (LSDs) (P 0.05) were used to compare genotype means for each cell type. Vacuolar K^+ concentrations in the mesophyll cells were not significantly different between Franklin and Wollaroi (P=0.05), but in the epidermal cells, Franklin was significantly lower than Wollaroi (P=0.05) by about 2 times. In Franklin, vacuolar concentrations approached zero at bulk leaf concentrations of about 70 mM (Figure 4.4B), indicating that preferential partitioning of K^+ to the mesophyll was greater in Franklin than in Wollaroi.

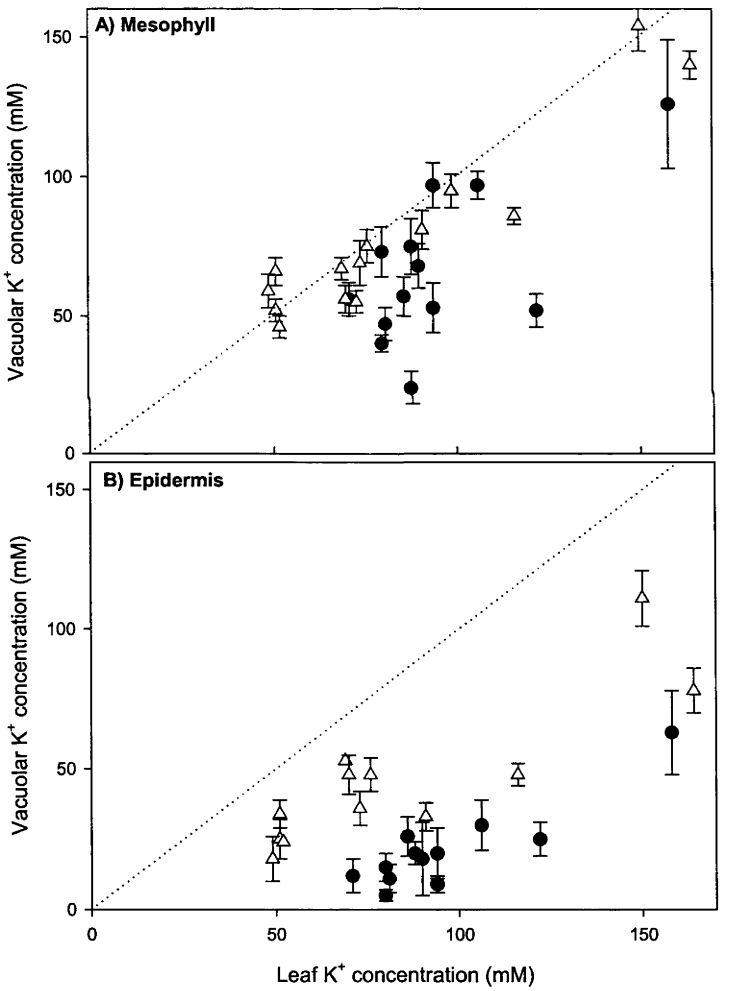

Figure 4.4 Relationship between bulk leaf K^+ concentration and K^+ concentration in the vacuoles of A) Mesophyll and B) Epidermal cells of leaf 3 of salt-stressed barley cultivar Franklin (●) and durum wheat cultivar Wollaroi (△). Vacuolar concentrations are given as means ± s.e. from 6 – 15 individual cells analysed by cryo-SEM X-ray microanalysis. Dotted line indicates 1:1 line.

Vacuolar Cl^- concentrations in both the mesophyll and epidermis increased linearly and proportionally with increasing leaf Cl^- concentration (above about 100 mM) in Franklin and Wollaroi (data not shown). Cl^- accumulated preferentially in the epidermal vacuoles to a similar extent in both Franklin and Wollaroi (Figure 4.5). The degree of partitioning to the epidermis was generally highest (~5-fold) at lower leaf Cl^- concentrations ($\leq$ 200 mM), but lessened to about 2-fold as bulk leaf Cl^- concentrations increased.

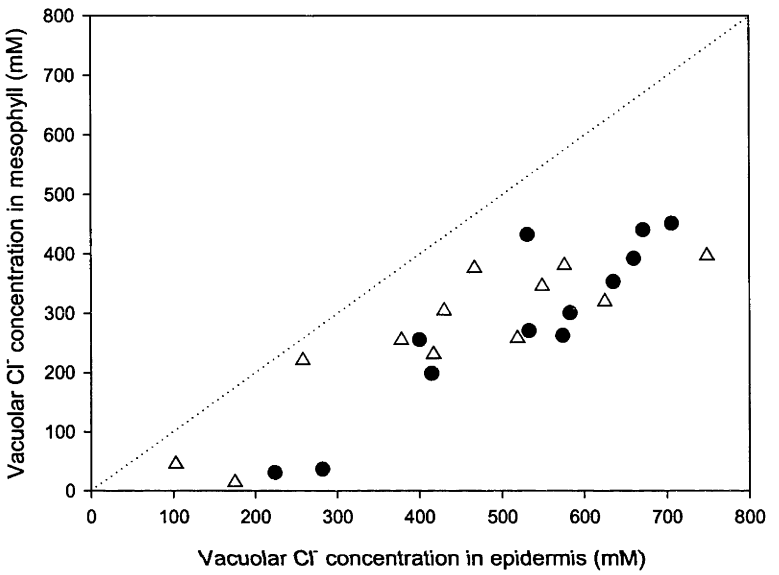

Figure 4.5 Distribution of vacuolar Cl^- in leaf epidermal and mesophyll cells of leaf 3 salt-stressed barley Franklin (●) and durum wheat Wollaroi (△). Vacuolar concentrations are given as means from 6 – 15 individual cells analysed by cryo SEM X-ray microanalysis. Dotted line indicates 1:1 line.

4.3.3.2 Na^+ and K^+ concentration in the cytoplasm of mesophyll cells

The relationship between bulk leaf Na^+ concentration and the calculated cytoplasm Na^+ concentration in Wollaroi and Franklin is summarised in Figure 4.6A. According to the calculations, the Na^+ concentration in the cytoplasm of Wollaroi steadily increased from about 150 mM to about 400 mM with increasing bulk leaf Na^+ concentration from 125 mM to 300 mM. Franklin was able to maintain the Na^+ concentration in the cytoplasm to between 150 – 200 mM with bulk leaf concentrations up to about 300 mM. Na^+ in the cytoplasm increased to very high concentrations (400 – 450 mM) with bulk leaf Na^+ concentrations of 350 - 400 mM.

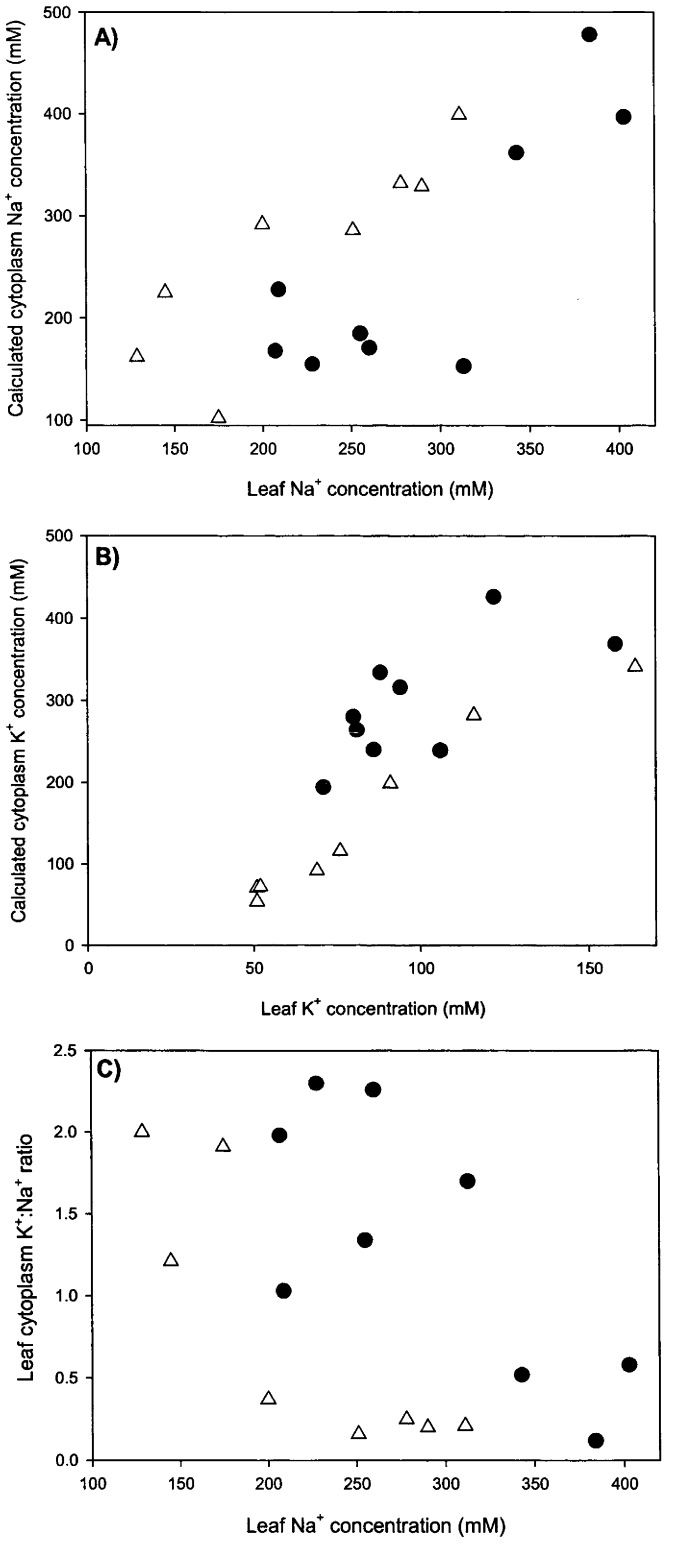

Figure 4.6 Relationships between A) the concentration of Na^+ in the bulk leaf tissue and the calculated Na^+ concentration in the cytoplasm; B) the concentration of K^+ in the bulk leaf tissue and the calculated K^+ concentration in the cytoplasm; and C) between leaf Na^+ concentration and the calculated K^+:Na^+ ratio in the cytoplasm of Franklin (●) and Wollaroi (△).

The calculated K^+ concentration in the cytoplasm ranged from between 200 and 400 mM in Franklin and 50 – 300 mM in Wollaroi (Figure 4.6B). The range of K^+ concentrations was a consequence largely of the level and duration of the salt stress, with K^+ being inversely proportional to the concentration of Na^+ in the leaf. A net result of lower vacuolar K^+ concentration in Franklin than Wollaroi (Figure 4.4) was higher cytoplasm K^+ concentrations in Franklin over a large range of bulk leaf K^+ concentrations (60 – 160 mM). At a bulk leaf K^+ concentration of about 70 - 80 mM, cytoplasm K^+ concentrations in Wollaroi were about 120 mM, i.e. half of that of Franklin, and fell to about 75 mM at leaf K^+ levels of 50 mM. Franklin was able to maintain cytoplasm K^+ at levels not lower than 200 mM, although bulk leaf concentrations in Franklin never fell below about 80 mM.

Efficient cellular and sub-cellular partitioning of both Na^+ and K^+ in salt-stressed Franklin barley led to the preservation of a favourable K^+:Na^+ ratio in the cytoplasm at high leaf Na^+ concentrations. The average K^+:Na^+ ratio calculated in the cytoplasm of Franklin was 1.8 (Figure 4.6C) at leaf Na^+ concentrations between 200 – 300 mM. This is in contrast to Wollaroi with an average K^+:Na^+ ratio of 0.3 for the same range in leaf Na^+ concentration. The cytoplasmic K^+:Na^+ ratio in Franklin dropped to about 0.4 at leaf Na^+ concentrations above 300 mM.

4.3.4 Relationship between photosynthetic capacity and leaf Na^+ concentration

By controlling Ci whilst measuring CO_2 assimilation rates at a low VPD (~ 1.0 KPa), the non-stomatal effects (e.g. biochemical limitations) of salinity could be directly ascertained. Figure 4.7 shows the relationships between leaf Na^+ concentration and two photosynthetic capacity parameters derived from A:Ci curves – V_{cmax} and J_{max}.

The photosynthetic capacity of the salt-treated plants with lower Na^+ concentration (mainly from the 25 mM NaCl pre-treatment) was about 25% higher than both Franklin and Wollaroi grown in 0 mM NaCl (Figure 4.7). This was presumably due to the osmotic effects of all salt treatments giving smaller and thicker leaves and subsequently, increasing the concentration of chloroplasts per unit leaf area. In support of this, the average specific leaf area (SLA) of salt-treated leaves of Franklin and Wollaroi was 255 and 258 cm^2 gDW^{-1} respectively, compared to the average SLA from control leaves of 297 and 292 cm^2 gDW^{-1}. Maximum photosynthetic capacity rates of salt-stressed plants appeared to be similar for both genotypes, with V_{cmax} at 120

– 140 μmol m^{-2} s^{-1} and J_{max} 180 – 200 μmol m^{-2} s^{-1}.

Both photosynthetic capacity parameters began to decline in a gradual linear manner at leaf Na^+ concentrations between 150 - 200 mM in Wollaroi, falling to about 60% of maximum at a leaf Na^+ concentration of 400 mM (Figure 4.7). In contrast, the photosynthetic capacity in Franklin did not begin to decline until a leaf Na^+ concentration of about 300 mM, but then declined at a similar rate to Wollaroi.

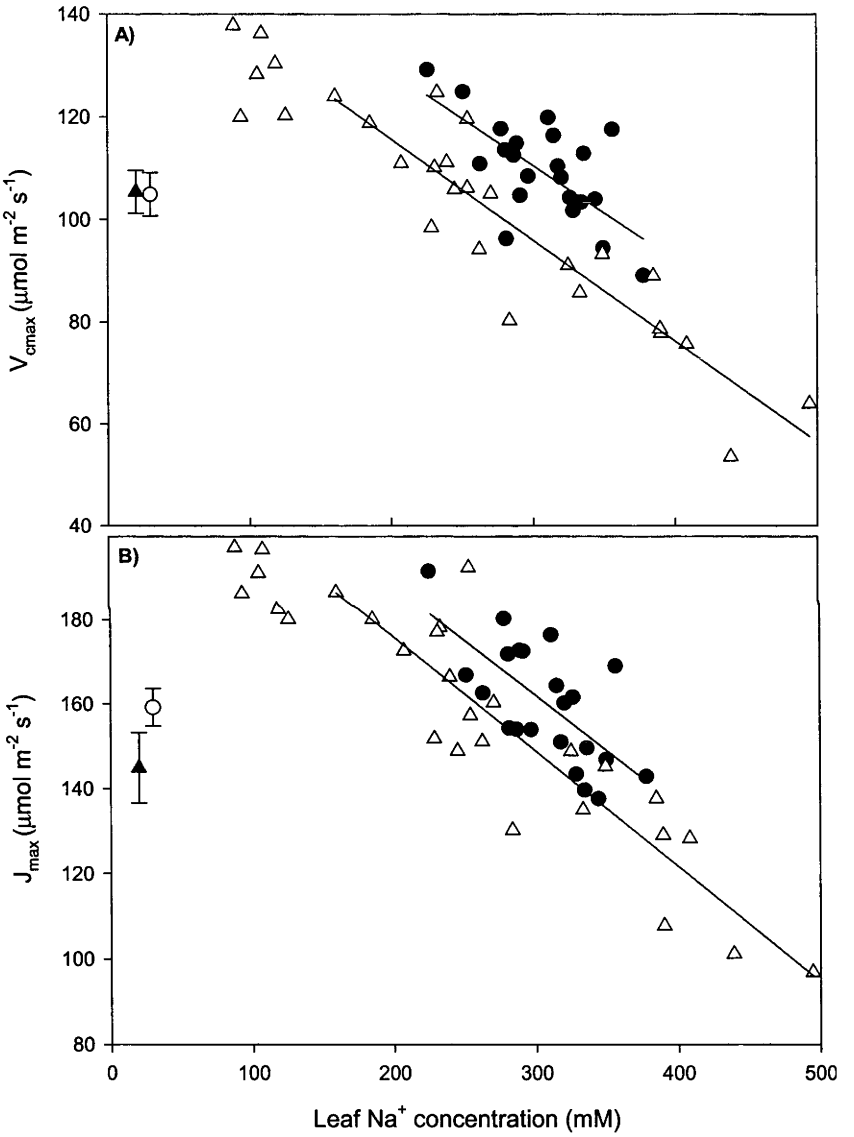

Figure 4.7 Relationship between bulk leaf Na^+ concentration and photosynthetic capacity parameters; A) V_{cmax} and B) J_{max} of leaf 3 of salt-stressed barley Franklin (●) and Wollaroi (△). Other symbols (○,▲) represent mean photosynthetic capacity ± s.e. (n=8) of controls of Franklin and Wollaroi respectively. A general linear model was used to fit a regression line to data with Na^+ concentrations above 150 mM for each genotype. The resulting F-test indicated no difference between slopes on each graph, while the difference between the intercepts was significant ($P<0.001$).

The relationship between photosynthetic capacity and leaf Cl^- concentration was not so clear. In Franklin there was no significant correlation between Cl^- concentration and capacity (r^2 = 0.20). In Wollaroi, while quite variable, photosynthetic capacity began to decline at leaf Cl^- concentrations over 300 mM (data not shown).

The relationship of declining capacity with increasing leaf Na^+ concentration appeared similar for both V_{cmax} and J_{max} indicating a tight coupling between the two capacity parameters (Figures 4.7, 4.8). V_{cmax} incorporates not only an estimate of photosynthetic capacity that is related to Rubisco activity, but also any limitations that may come from diffusional resistance to CO_2 transfer in the mesophyll. It was unlikely that mesophyll resistance was the cause of genotypic differences in the relationship between Na^+ concentration and photosynthetic capacity as both genotypes had identical photosynthetic capacity (V_{cmax}) under control conditions, maximum V_{cmax} rates were similar (at lower leaf Na^+ concentrations) and the ratio of V_{cmax} to J_{max} was unchanged at about 1.5 for both genotypes throughout the broad range of leaf Na^+ concentration (Figure 4.8).

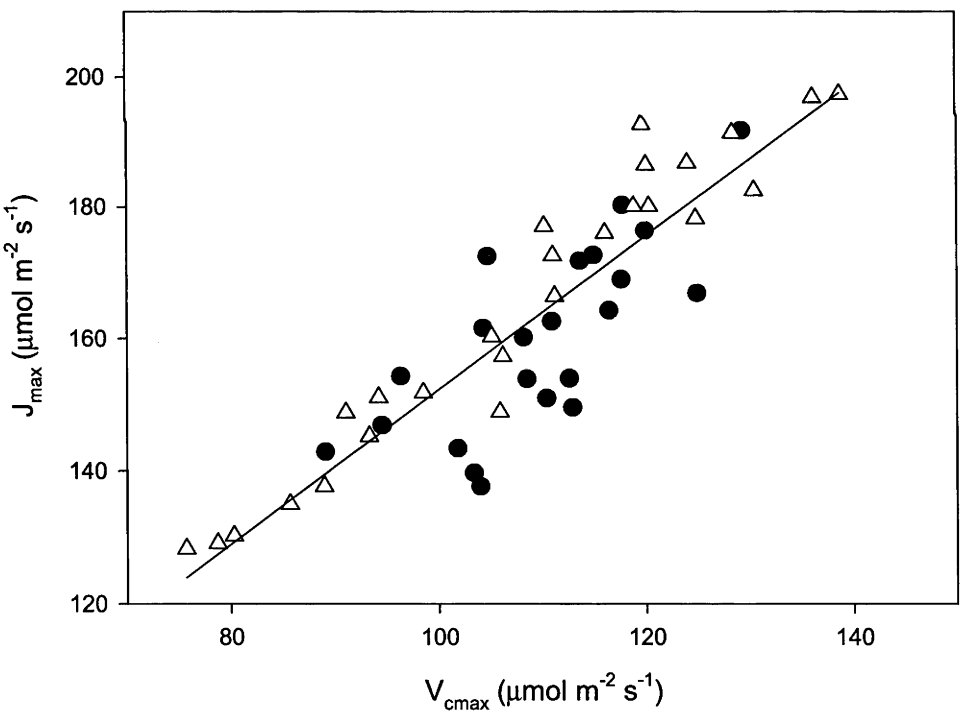

Figure 4.8 Relationship between V_{cmax} and J_{max} of Franklin (●) and Wollaroi (△). Photosynthetic capacity parameters determined on leaf 3 of salt-stressed seedlings. Fitted linear regression is described by the following equation: y = 1.2x + 35.6 (r^2 = 0.81).

4.4 DISCUSSION

Efficient cellular and sub-cellular partitioning of Na^+ and K^+ in Franklin barley led to the preservation of a favourable K^+:Na^+ ratio in the cytoplasm to higher leaf Na^+ concentrations in comparison with Wollaroi durum wheat. Photosynthetic capacity of Franklin barley declined at higher leaf Na^+ concentrations than in Wollaroi. The maintenance of photosynthetic capacity in Franklin was associated with the maintenance of higher K^+, lower Na^+ and the resulting higher K^+:Na^+ in the cytoplasm of mesophyll cells.

This study used tissue freezing techniques combined with cryo-SEM X-ray microanalysis to determine ion concentrations in a range of cell types and compartments. Irrespective of the sampling technique used, measuring sufficient cells across the spectrum of cellular locations to give an accurate representation of vacuolar contents of a particular cell type will always be problematic due to the distinctive constraints of the various techniques and also because cells are inherently heterogenous. All the techniques used in previous studies have their own specific limitations and possible sources of error. For example, Fricke et al. (1994; 1996) acknowledged that there was likely to be a 10% error associated with sampling mesophyll vacuoles using a microcapillary through contamination from the cytoplasmic compartment. Likewise, over or under-etching the cryo-planed leaf face for X-ray microanalysis will also over or under estimate vacuolar ion concentrations. In this study we attempted to sample as many cells as feasible (typically 10 – 12) for each cell type across a range of cellular locations. To this end, the sum of vacuolar ion concentrations (Na^+, Cl^-) approximated leaf concentrations and were generally similar to those measured in other studies (eg. Fricke et al., 1996; Flowers and Hajibagheri, 2001).

Similarly, the calculated K^+ concentration of the cytoplasm in the leaves of both the barley and durum wheat at leaf K^+ concentrations above 80 mM (200-350 mM), was at least twice as high (see Section 4.4.3) than that reported in Cuin et al. (2003). There is a likelihood of errors because the cytoplasmic concentrations were calculated from the difference between two large numbers, and a possibility that our cytoplasmic concentrations are over-estimated because of the assumptions used in the calculation. For example, Na^+ and K^+ concentrations were assumed to be the same in the cytosol and chloroplasts. If K^+ was partitioned preferentially into the chloroplasts by a factor of 2:1

and as chloroplasts make up about 70% of the cytoplasm volume in mesophyll cells, cytosolic K^+ concentrations would be halved.

4.4.1 Partitioning of Na^+ and K^+ between mesophyll and epidermis cells

Na^+ was distributed equally between the mesophyll and epidermis in Wollaroi, with the Na^+ concentration in both cell types increasing as leaf Na^+ increased. However, Franklin appeared to have a greater capacity for storage of Na^+ in mesophyll vacuoles, partitioning slightly greater Na^+ (~10%) to the mesophyll compared to Wollaroi. Also using X-ray microanalysis, Huang and van Steveninck (1989) found similar concentrations of Na^+ in the epidermis and mesophyll of two barley cultivars (differing in salt tolerance) grown for 1 d in 50 and 100 mM NaCl, but twice the Na^+ in the mesophyll of both genotypes after 4 d in 50 mM NaCl. Similarly, using the same technique, Leigh and Storey (1993) encountered more mesophyll than epidermal cells with detectable levels of Na^+. However, studies using different techniques have offered contrasting results. Karley et al., (2000a) measured 10 fold higher Na^+ concentrations in isolated protoplasts from barley epidermal cells (41 mM) than from mesophyll cells (3 mM). These measurements were taken from leaves of non salt-stressed plants and are at odds with Dietz et al. (1992) who, using the same technique, found similarly low levels in protoplasts from both mesophyll and epidermal cells. Using a microcapillary to extract sap from single cells, Fricke et al. (1996) generally found higher Na^+ concentrations in epidermal vacuoles than mesophyll vacuoles of salt-stressed barley seedlings. This discrepancy may have resulted from the exclusive sampling of mesophyll cells from a particular cellular location (cells lining the stomatal cavity), as the microcapillary was inserted through the stomatal pore. The diversity of results summarised in the above mentioned studies, probably reflects the range of techniques and experimental conditions used.

In contrast to the relatively uniform pattern of Na^+ distribution, there was preferential partitioning of K^+ to the mesophyll evident in both genotypes. The preferential partitioning of K^+ to the mesophyll found in Franklin is consistent with other studies using barley under similar levels of salinity (Leigh and Storey, 1993; Fricke et al., 1996; Cuin et al., 2003). Durum wheat (Wollaroi) has not previously been examined, but appears to follow a similar general pattern to barley in the intercellular distribution of inorganic ions in salt-stressed leaves. Significant differences between the barley and durum cultivars were also apparent, however. Perhaps most noteworthy was

the degree of partitioning of K^+ between epidermal and mesophyll cells. Wollaroi maintained 2 – 3 times more K^+ in epidermal vacuoles than Franklin, whereas the difference was much less pronounced in vacuoles of mesophyll cells. Epidermal cells could be considered as feasible ion storage compartments to safeguard more physiologically important cells, such as the mesophyll cells, from the potentially toxic effects of Na^+ and Cl^- in high concentrations. How more K^+ was partitioned to the vacuoles in the epidermis in Wollaroi than Franklin remains unclear. Karley et al. (2000b) summarise possible transport mechanisms. This partitioning was likely to have had a strong influence on the K^+ concentrations in the cytoplasm of mesophyll cells, which were lower for Wollaroi than for Franklin (Figure 4.6B).

4.4.2 Preferential partitioning of Cl^- between mesophyll and epidermal cells

Preferential partitioning of Cl^- to epidermal vacuoles in both durum wheat and barley, particularly at low leaf Cl^- concentrations (50 – 150 mM), is consistent with the findings of other studies using salt-stressed barley (Huang and van Steveninck, 1989; Leigh and Storey, 1993, Fricke et al., 1996). This reproducible pattern of epidermal Cl^- deposition has also been established in non-saline conditions (Huang and van Steveninck, 1989; Fricke et al., 1996), and additionally, Karley et al. (2000a) found significant Cl^- partitioning in the isolated epidermal protoplasts of young barley leaves in control conditions. Leigh and Tomos (1993) concluded that the distribution of Cl^- into the epidermis was a likely result of preferential Cl^- movement through the vein extension apoplast. Higher Cl^- concentrations in the epidermal cells immediately above and around lateral veins compared to epidermal cells in between veins, observed by Fricke et al. (1995), would tend to support this hypothesis.

Chloride was measured at low concentrations (< 40 mM) in the vacuoles of mesophyll cells of both Franklin and Wollaroi at low tissue concentrations, and while it increased with increasing tissue concentrations above 200 mM, epidermal vacuoles still accumulated about twice the amount of Cl^- as mesophyll vacuoles. Efficient partitioning of Cl^- to the epidermis would keep Cl^- concentrations lower in the mesophyll vacuole and possibly at lower or non-toxic concentrations in the cytoplasm. Fricke et al. (1996) also found epidermal Cl^- concentrations 3 – 3½ times higher than in the mesophyll in the fully expanded (3^{rd}) leaf from barley grown in 100 and 150 mM NaCl. They concluded that the relatively low Cl^- concentration in the mesophyll (vacuoles) of between 120 – 170 mM would be unlikely to affect photosynthetic processes. A lack of

genotypic differences in Cl^- partitioning between the salt-tolerant Franklin barley and the salt-sensitive Wollaroi in this current study would also indicate that Cl^- was not responsible for the decline in photosynthetic capacity. At higher tissue concentrations (300 – 400 mM), vacuolar compartmentation may eventually break down leading to higher and potentially toxic Cl^- concentrations in the cytoplasm. In support of this, Cl^- concentrations of 250 – 300 mM, which affected the efficiency of RuBP carboxylase, were found in the cytoplasm, chloroplasts and in the vacuole of salt-stressed *Phaseolus*, indicating a breakdown in vacuolar compartmentation (Seemann and Critchley, 1985).

4.4.3 Na^+ and K^+ and K^+:Na^+ ratio in the cytoplasm

The toxicity of high Na^+ concentrations in the cytoplasm mostly relates to its ability to compete with K^+ for protein binding sites. High Na^+:K^+ ratios will therefore inevitably lead to the disruption of many important K^+-dependent enzymatic and physiological processes, which ultimately affect growth (Maathuis and Amtmann, 1999; Tester and Davenport, 2003). Earlier, Leigh and Wyn Jones (1984) postulated a requirement for the maintenance of critical K^+ concentrations in the cytoplasm with declining vacuolar and tissue K^+ concentrations, a scenario which is brought about by the presence of medium to high salinity levels. My results show evidence for genotypic variation in this ability to maintain high K^+ and an adequate K^+: Na^+ ratio in the cytoplasm of salt-stressed plants. The concentration of K^+ in the cytoplasm of Franklin (barley) was generally maintained above 200 mM with declining leaf tissue concentrations, which was in contrast to Wollaroi (durum wheat), where cytoplasmic K^+ declined in parallel with vacuolar and tissue concentrations (Figures 4.4 and 4.6B). The Franklin (barley) results are consistent with other observations. Walker et al. (1996), using a triple-barrelled microelectrode, established that while K^+ activities (a_K) of epidermal and cortical vacuoles in the roots of barley decreased proportionally with K^+ availability, cytosolic a_K remained relatively constant (between 70-75 mM) until the root K^+ concentration decreased to 25 mM. Using the same procedure, similar cytosolic a_K values were also found in mesophyll cells of barley grown in 0 and 200 mM NaCl (Cuin et al., 2003).

Na^+ was maintained at about 150 mM in the cytoplasm of Franklin with increasing leaf Na^+ up to 300 mM, which was in contrast to Wollaroi, where Na^+ in the cytoplasm generally paralleled vacuolar and leaf tissue concentrations (Figure 4.3,

4.6A). With the maintenance of high K^+ and medium Na^+ concentrations in the cytoplasm, salt-tolerant Franklin was able to sustain a K^+:Na^+ ratio in the cytoplasm of about 1.8 (Figure 4.6C), which was 6 – 8 times higher than that for the salt-sensitive Wollaroi. In the only other study of this kind, Carden et al. (2003), using a Na^+-selective microelectrode, found similar results between two barley varieties differing in salt tolerance when grown in 200 mM NaCl. Salt-tolerant barley variety Gerbel was more effective at excluding Na^+ from the cytosol and sequestering it in the vacuoles of root cortical cells compared to the salt-sensitive variety Triumph. Gerbel was also better at maintaining cytosolic a_K which resulted in a 10 fold increase in the cytosolic K^+:Na^+ over Triumph after 5 d in a high salinity. Flowers and Hajibagheri (2001) concluded that Na^+ (and Cl^-) cytoplasmic concentrations estimated at between 257 – 350 mM in root cortical cells of Gerbel and Triumph would be sufficient to reduce enzyme activity *in vitro*. Interestingly, the vacuolar compartmentation of Na^+ broke down in Gerbel after 8 d, resulting in similar cytosolic K^+:Na^+ ratios to those in Triumph. Similarly, in the present study, cytoplasmic Na^+ in Franklin rapidly increased at leaf concentrations higher than 300 mM, resulting in a 4 fold decrease in the K^+:Na^+ ratio, indicating a similar break down in vacuolar compartmentation.

4.4.4 Cytoplasmic Na^+ and K^+ concentrations and photosynthetic capacity

The significance of maintaining high K^+, low Na^+ and the resulting favourable K^+:Na^+ ratio in the cytoplasm of Franklin barley can be seen in the genotypic differences in the relationship between the onset of a decline in photosynthetic capacity and leaf Na^+ concentration. The decline in photosynthetic capacity corresponded with leaf Na^+ concentrations between 150 – 200 mM in Wollaroi, which related to the cytoplasm Na^+ concentrations increasing to between 200 – 300 mM and a decrease in the K^+:Na^+ ratio from 1.7 to 0.24. In contrast, photosynthetic capacity in Franklin declined with leaf Na^+ approaching about 300 mM. This also corresponded to cytoplasmic Na^+ increasing to above 300 mM and the K^+:Na^+ ratio decreasing from 1.8 to 0.5.

Accumulation of Na^+ or Cl^- in the leaf and poor compartmentation in the vacuoles have been suggested as the main causes of a reduction in photosynthesis of salt-stressed plants, through a direct toxic ion effect either on the photosynthetic machinery (Yeo et al., 1985) or on key enzymes involved in carbon fixation (Greenway and Munns, 1980; Seemann and Critchley, 1985; Tester and Davenport, 2003). Others have attempted to link CO_2 assimilation rates with leaf ion contents of salt–affected

plants (Fricke et al., 1996, Sibole et al., 2003). The problem with this approach is that it fails to exclude the stomatal component of reduced CO_2 assimilation rate that is largely due to the osmotic component of salt stress. By measuring photosynthetic capacity parameters (V_{cmax}, J_{max}) derived from A:Ci curves, stomatal limitations on photosynthesis are removed as a factor, leaving only biochemical and possibly diffusional (mesophyll resistance to CO_2 diffusion) limitations to be considered.

Photosynthetic capacity in both cultivars began to decline at cytoplasmic Na^+ concentrations of about 300 mM. For Wollaroi this was reached at a tissue Na^+ concentration of about 200 mM, whereas for Franklin the same cytoplasmic concentration was reached at about 300 mM tissue Na^+. The importance of this genotypic difference between Franklin and Wollaroi in the decline in photosynthetic capacity at high leaf Na^+ concentration should not be under-estimated. Assuming Na^+ concentrations in leaves increase at a rate of about 5-10 mM per day (Munns, 1993) for both genotypes, the difference in time between having 200 mM (Wollaroi) and 300 mM (Franklin) Na^+ in the leaf is likely to be in the order of 10 to 20 days. This would give Franklin a further 10 to 20 days of optimal carbon acquisition for a given leaf, notwithstanding the stomatal limitations which are likely to be common to both genotypes. The extra carbon fixed would prolong the supply of assimilate to growing regions and promote tiller initiation and growth in young seedlings (Nicolas et al., 1993), and would also prolong the supply of assimilate to the growing ears and filling grains of adult plants, increasing grain number, grain size, and yield (Husain et al., 2003).

Chapter 5

TOLERANCE TO OSMOTIC STRESS IN SALT-STRESSED DURUM WHEAT

5. TOLERANCE TO OSMOTIC STRESS IN SALT-STRESSED DURUM WHEAT

5.1 INTRODUCTION

Stomatal and non-stomatal factors limit photosynthesis of salt-stressed plants. Stomatal conductance is more sensitive to salinity than the non-stomatal components of photosynthesis and imposes a limitation on photosynthesis over the duration of the stress.

In a previous study with two tetraploid wheats (Chapter 3), stomatal conductance was reduced immediately with the onset of salinity and was the initial and most profound cause of a decline in CO_2 assimilation rate (James et al., 2002). Timing is important in separating the relative impact of the two factors. Stomatal factors occur immediately with the onset of salt-stress (Fricke et al., 2004) and continue over the course of the stress, whereas non-stomatal factors occur over the timeframe of weeks to months with the build up salts in the older leaves (Munns, 1993; 2002; James et al., 2002). One way to test for variation in tolerance to the osmotic stress component of salt-stress may be to measure changes in stomatal conductance on plants before salts build up in the leaf.

It is clear that there is genotypic variation in the salt tolerance of crop plants such as wheat associated with leaf death due to high salt concentrations in the leaves (Gorham, 1990; Munns et al., 1995). Less clear and largely untested is whether there is genotypic variation in salt tolerance associated with tolerance to osmotic stress (Neumann, 1997). It is possible that the salt-induced decline in CO_2 assimilation rate could be reduced if stomata were less sensitive to the osmotic component of salt stress.

Genotypic variation in intrinsic stomatal conductance without stress has been reported for irrigated or well watered wheat (Condon et al., 1990; Morgan and LeCain, 1991; Fischer et al., 1998) and barley (e.g. Isla et al., 1998), and under these conditions where water is not limiting, stomatal conductance appeared to be positively correlated with grain yield. Under water limiting conditions such as drought and salinity the relationship between stomatal conductance and yield is less clear, with an apparent compromise between the requirement for CO_2 uptake to maintain photosynthesis (and growth) and the consequent water loss associated with open stomata. Carbon isotope

discrimination (Δ) has been used as an integrated measure of photosynthetic performance in response to environmental factors such as water availability and VPD and as a predictor of genotypic differences in water use efficiency (Farquhar and Richards, 1984). Under saline conditions Δ has been found to decrease in wheat (Rivelli et al., 2002), barley (Shen et al., 1994; Isla et al., 1998) and in rice (Shaheen and Hood-Nowotny, 2005), largely due to a decrease in stomatal conductance and to a lesser extent, an increase in photosynthetic capacity. There have been some studies relating stomatal conductance or Δ to salt tolerance in cereals, but these have produced conflicting results. Stomatal conductance and Δ were not found to be useful physiological traits when screening for salt tolerance in barley (Isla et al., 1998) or wheat (Jiang et al., 2006a); however, Δ correlated well with a visual score for salt tolerance in rice seedlings (Shaheen and Hood-Nowotny, 2005).

The aims of this study were therefore firstly, to assess genotypic variation in the response of stomatal conductance to salt stress in a large collection of durum wheat cultivars and landraces; and secondly, to examine the relationship between genotypic differences in salt-induced reductions in stomatal conductance and genotypic differences in relative growth rate and CO_2 assimilation rate. It is possible that salt-induced osmotic stress affects both stomatal conductance and growth similarly. Therefore a third objective of this study was to test if the response of stomatal conductance could provide a quick and reliable way to select for better growth under saline conditions.

These aims were contingent on the ability to measure the effects of osmotic stress on stomatal conductance, without the complication of salinity-induced changes in leaf morphology (such as a decrease in area and an increase in leaf thickness and therefore an increase in stomatal density and photosynthetic capacity), and before a build up of salt in the leaves could affect CO_2 assimilation rate. Stomatal conductance measurements were therefore taken on a leaf that was already fully expanded before the salt stress commenced, at 5 – 6 d after the salt treatment started so that salt concentrations were low, and compared to leaves of the same age without salt treatment.

5.2 MATERIALS AND METHODS

5.2.1 Germplasm

A total of 50 genotypes listed in Appendix 5.1 were screened for stomatal conductance. This included a selection of 20 *Triticum turgidum* L. landraces (from the collection of 47 landraces screened in Chapter 2 for Na^+ tissue tolerance), including five putative tissue tolerant selections (Line 414, Line 528, Line 139, Line 362 and Line 255) and a selection of 25 durum cultivars from a range of locations, sourced from the Australian Winter Cereals Collection. The current Australian durum cultivars Tamaroi, Wollaroi and Bellaroi were also included with a representative Australian bread wheat cultivar Westonia, barley cultivar Franklin, and well known Indian salt-tolerant wheat landrace Kharchia.

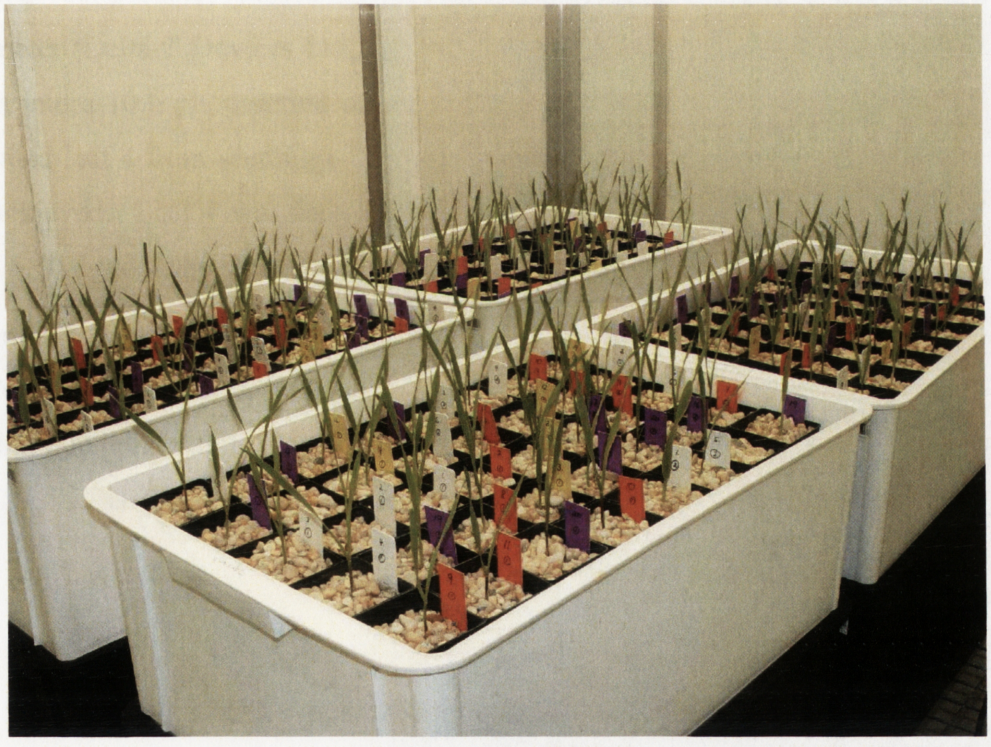

Figure 5.1 Layout of hydroponic trays in controlled growth chamber. Seedlings are at the leaf 2.0 stage, about 8 d before the commencement of the salt treatment

5.2.2 Growth conditions

Plants were grown in supported hydroponics in 6.5cm square x 15.8 cm deep pots containing quartz gravel in 40 L trays containing 40 pots in ½ modified Hoagland's solution (Figure 5.1). At approximately 14 d after emergence (DAE), 25 mM NaCl was added twice a day to a final concentration of 150 mM, and $CaCl_2$ was added to give a

final concentration of 10 mM. Plants in a control treatment were grown in separate trays in ½ modified Hoagland's solution. Plants were grown in a controlled environment chamber with a 10 h photoperiod and PPFD of 800 μmol m^{-2} s^{-1} at 25°C during the day and 18°C during the night.

5.2.3 Experimental series

Stomatal conductance screen of 50 genotypes (Experiments 1 - 3)

Genotypic variation in stomatal conductance of salt-stressed durum cultivars and landraces was examined in three separate screening experiments, each containing 20 genotypes grown in control and salt treatments as listed in Appendix 5.1

Stomatal conductance measurements were obtained using a Delta–T AP4 cycling porometer (Delta-T Devices Ltd, Burwell, UK). Measurements were taken between 3 to 7 h into a 10 h photoperiod on the abaxial surface, from the mid-portion of a fully expanded leaf 4 from seedlings 19 – 21 DAE, 3 – 5 d after the final salt concentration was achieved. Leaf 4 was fully expanded at the commencement of the salt treatment and 8 – 10 d old when stomatal conductance was measured.

Preliminary experiments found that while taking stomatal conductance measurements, the CO_2 concentration in the controlled environment chamber increased from 380 ppm to about 600 ppm within 15 min (Figure 5.2), due to expired air from the operator.

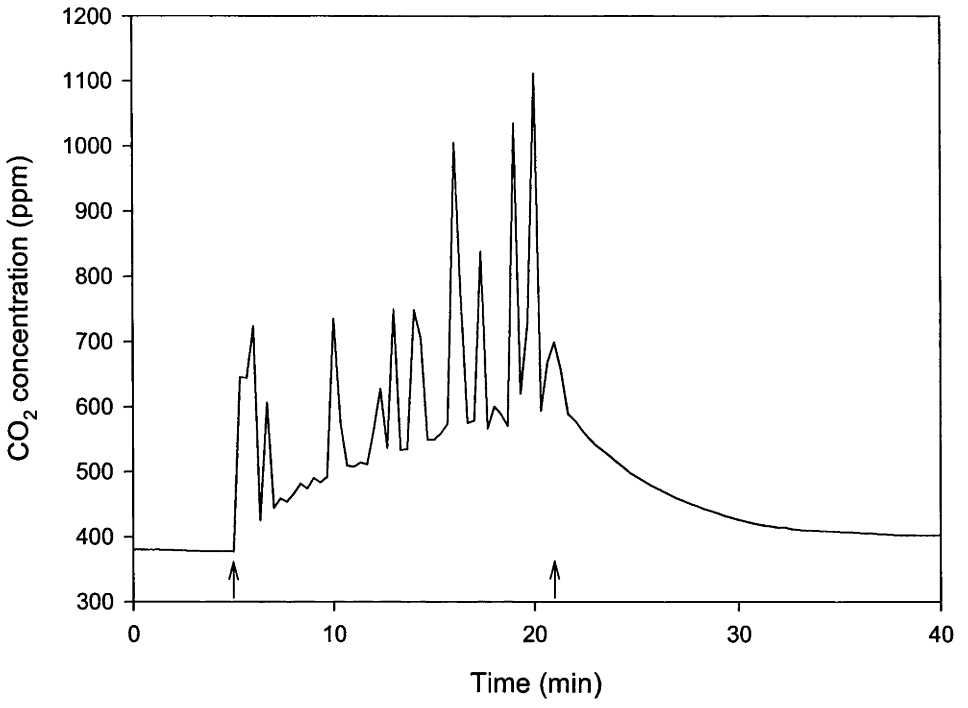

Figure 5.2 Increase in CO_2 concentration (ppm) in controlled environment chamber during 15 min of measurements without exhausting tube. Arrows indicate the start and end of a typical screening run.

To maintain CO_2 levels at ambient, a face mask with exhausting tube to the outside of the chamber was used when conducting stomatal conductance measurements. CO_2 concentration in the chamber under these conditions did not exceed 400 ppm.

Stomatal conductance of 16 selected large and small response lines (Experiment 4)

To validate the response status of lines with the greatest and least response of stomatal conductance in 150 mM NaCl from Experiments 1 – 3, eight putative large response and small response durum cultivars were retested. Plants were grown in salt and control conditions and g_s measured on leaf 4 as described above. The blades of leaf 4 were harvested immediately after stomatal conductance measurements (11 d old) and were dried at 70°C for 3 days, weighed, extracted in 500 mM HNO_3 at 80°C for 1.5 h and analysed for Na^+ by an Inductively Coupled Plasma – Atomic Emission Spectrometer (Vista Pro, Varian, Melbourne, Australia). Chloride analysis was carried out using the same extracts with a specific ion (Cl^-) electrode (Model 96-17, Orion, Cambridge MASS, USA).

Relative growth rates and gas exchange measurements of 6 selected large and small response lines (Experiment 5)

Three small g_s response durum cultivars (Seklavi, Coulter, Hercules) and three large g_s response durum cultivars (Durex, Candicans, Koelz W3158), selected from Experiment 4, together with current durum cultivars Tamaroi and Wollaroi were tested to determine if extremes in g_s response impacted on CO_2 assimilation rate (A) and also to determine the relationship between g_s and relative growth rate (RGR). Plants were grown in control and salt treatments as described above. Stomatal conductance was measured as described above (~20 DAE), on leaf 4 (9 d old) on 6 reps of all genotypes.

Shoot harvests were taken of 3 replicate plants of each genotype for each treatment at three sampling times; 16, 21 and 26 DAE. The first sampling time corresponded to the day when the final salt concentration (150 mM NaCl) was reached. Shoots were cut just below the crown and dried at 65°C for 3 d. Shoot relative growth rate was calculated for each period as:

$$RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1)$$

Where W is total shoot dry weight and t is time in days at the start and finish of each period.

Measurements of A were made on mid portion of leaf 4 from 22 to 23 d old seedlings in a constant environment cabinet using a LI-6400 portable gas exchange system (LI-COR, Lincoln NE, USA). Leaf 4 was fully expanded at the commencement of the salt treatment and 12 – 13 d old when A was measured. All measurements were taken between 3 to 7 h into a 10 h photoperiod and settings were chosen to match the cabinet conditions. Leaf temperature was maintained at 25°C, light intensity was set at 800 μmol m^{-2} s^{-1} with a red/blue light source, ambient CO_2 (in leaf chamber) was set at 380 μbar and the leaf to air VPD maintained at 1.1 KPa.

5.2.4 Experimental design and statistical analysis

In Experiments 1 – 4, genotypes were randomly positioned in salt and control tanks according to a latinized spatial random experimental design with four replicates (example in Appendix 5.2). Prior to analysis, stomatal conductance data were subjected to a test for non-normality. For Experiments 1 - 3, residuals were not normal, so the data

was transformed using a square root function. The transformed data were analysed via a mixed linear model, fitted using the REML procedure in GenStat (GenStat Release 9.1 (Rothamsted Experimental Station). Genotype means and standard errors for the two treatments were obtained from the analysis. Confidence intervals for the ratio of salt mean to control mean for each genotype were obtained using Fieller's theorem (Finney, 1978). 83% confidence intervals were chosen for a comparison of the ratios, as these provide an approximate equivalent to a LSD at the 5% level of significance.

5.3 RESULTS

5.3.1 Stomatal conductance screens (Experiments 1 -3)

Variation in stomatal response to salinity-induced osmotic stress was assessed by measuring stomatal conductance (g_s) of about 50 durum wheat genotypes grown in a high salinity (150 mM NaCl), relative to control (non-saline) conditions in three separate screening experiments. Significant variation of between 2 to 3 fold in stomatal response to osmotic stress was found among genotypes across all three experiments (Figure 5.3). Stomatal conductance values are given in Appendix 5.3. On average, the high salinity treatment reduced g_s by about 40%, ranging between 60% (e.g. Durex, Candicans – 'large response' genotypes) to g_s values not significantly different from controls (e.g. Edmore, Seklavi – 'small response' genotypes). Stomatal conductance of control plants varied considerably (100 – 870 mmol m^{-2} s^{-1}) (Appendix 5.3). The stomatal response of most current Australian durum cultivars (Tamaroi, Wollaroi, Kalka) and hexaploid wheats (Westonia, Kharchia) was high to intermediate (g_s in salt, 40 – 65 % control), with only durum cultivar Bellaroi (81% of control) and barley cultivar Franklin (94% of control), showing a small g_s response in salt.

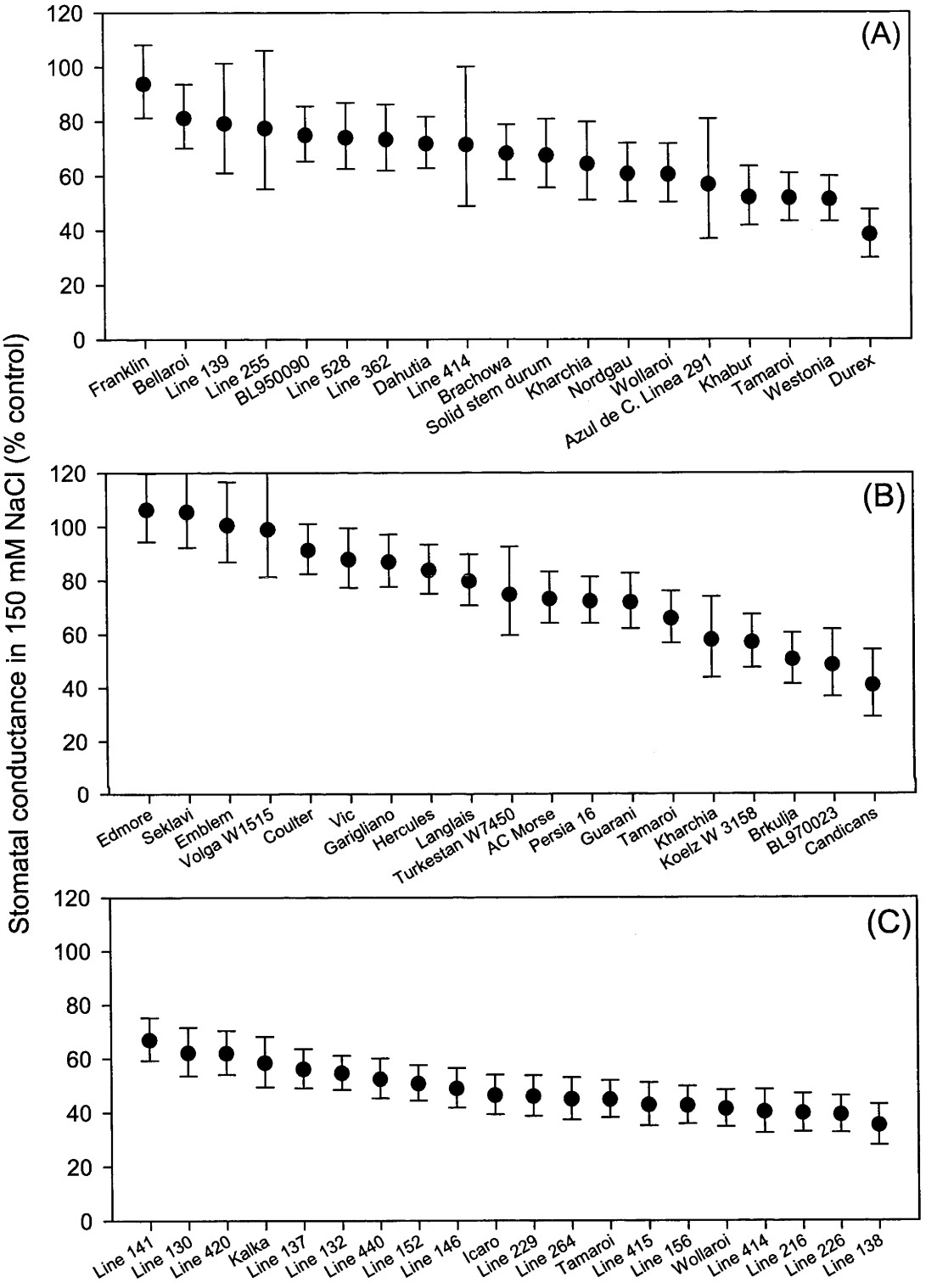

Figure 5.3 Stomatal response of 50 durum wheat genotypes with hexaploid wheat (Westonia, Kharchia) and barley (Franklin) to 150 mM NaCl in 3 screening experiments A) Exp 1, B) Exp 2 and C) Exp 3. Bars are equivalent to $LSD_{(0.05)}$.

5.3.2 Stomatal conductance of 16 selected large and small response lines (Experiment 4)

Eight lines with relatively low g_s and eight lines with relatively high g_s in salt, relative to their non-salt control in Experiments 1 – 3, were retested in a 4th experiment using identical conditions. These lines are listed in Table 5.1 and are called 'large response' lines and 'small response' lines, respectively.

Of the putative small response lines, at least half had high g_s in salt relative to their controls when retested. Stomatal conductance of these lines averaged about 70% and ranged between 55 – 83% of their controls (Table 5.1). Conversely, the eight putative large response lines averaged 46% of their controls when re-tested, but there was greater variation in g_s amongst these lines (26 – 70%). The current durum varieties were intermediate between the large and small response groups.

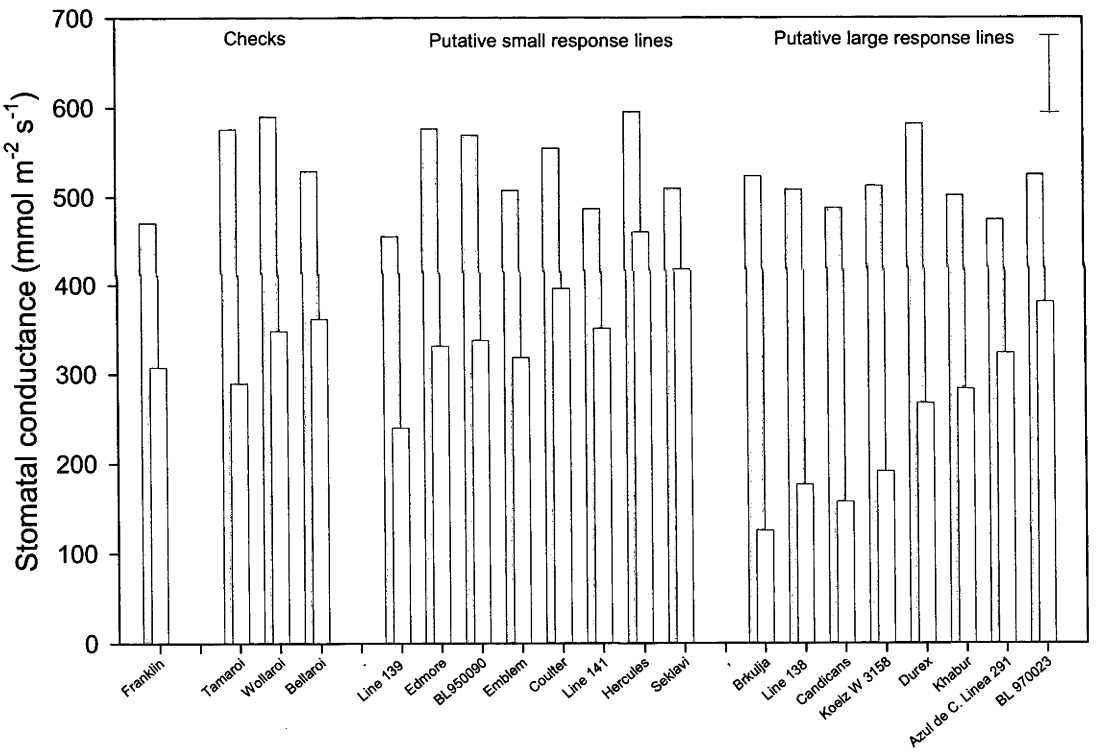

Figure 5.4 Stomatal conductance of 19 durum genotypes and Franklin (barley) grown in control (filled bars) and 150 mM NaCl (open bars) treatments. Bar indicates $LSD_{(0.05)}$.

There was no significant variation (P = 0.05) in the g_s of lines under control conditions (Figure 5.4). Average g_s for the small response lines was 531 mmol m^{-2} s^{-1} (range: 455 - 595) compared to 514 mmol m^{-2} s^{-1} (range: 474 - 582) for the large

response lines. This indicated that a higher g_s in salt was not a function of an intrinsically high g_s as measured under control conditions. The two lines with the greatest contrast in response to salt (Seklavi and Brkulja) (Table 5.1) had identical g_s under control conditions (Figure 5.4).

Table 5.1 Growth stage at start of salt treatment (14 DAE), stomatal conductance and Na^+ and Cl^- concentration in leaf 4 of 8 candidate small response durum lines, 8 candidate large response durum lines, 3 current durum varieties and barley cultivar Franklin, grown in 150 mM NaCl.

Category	Name	Growth stage (Zadok scale)	Stomatal conductance in salt (% control)	Leaf ion concentration (μmol gDW^{-1}) Na^+ mean ± s.e.	Cl^- mean ± s.e.
Barley cv.	Franklin	4.1	65.4	659 ± 41	1048 ± 43
Durum cvs.	Bellaroi	4.0	69.6	484 ± 39	758 ± 41
	Wollaroi	3.9	61.7	358 ± 17	755 ± 55
	Tamaroi	3.8	48.7	463 ± 38	789 ± 50
	Mean:		**60.0**	**435**	**767**
Small response lines	Seklavi	4.0	82.9	444 ± 19	621 ± 36
	Hercules	4.0	77.3	463 ± 18	694 ± 41
	Coulter	4.1	74.1	501 ± 37	624 ± 22
	Line 141	3.9	74.1	535 ± 16	737 ± 24
	Emblem	4.2	63.2	599 ± 28	803 ± 45
	BL950090	3.5	59.7	584 ± 29	765 ± 24
	Edmore	3.7	55.7	401 ± 17	819 ± 22
	Line 139	3.8	55.4	446 ± 26	679 ± 20
	Mean:		**67.8**	**497**	**718**
Large response lines	BL970023	4.0	70.5	402 ± 23	706 ± 33
	Azul de C.Line 291	4.2	69.0	381 ± 10	792 ± 11
	Khabur	4.0	58.3	367 ± 20	690 ± 16
	Durex	4.1	46.9	491 ± 18	744 ± 53
	Koelz W 3158	3.9	35.9	402 ± 23	706 ± 33
	Candicans	4.2	32.5	375 ± 28	758 ± 52
	Line 138	3.8	31.8	659 ± 5	915 ± 57
	Brkulja	3.5	25.6	554 ± 34	795 ± 16
	Mean:		**46.3**	**454**	**763**
$LSD_{(0.05)}$			19.2		

Growth stage assigned using Zadoks scale, (Zadoks, et al., 1974).

The Na^+ and Cl^- concentrations measured in leaf 4 (immediately after stomatal conductance measurements), varied little between genotypes and were considered to be non-toxic (Table 5.1). For example, the average Na^+ concentration was about 450 μmol gDW^{-1}, which is equivalent to about 80 mM on a leaf water basis. Similarly, the average Cl^- concentration was 750 μmol gDW^{-1}, which is equivalent to about 130 mM. There was no significant differences ($P = 0.05$) in mean Na^+ or Cl^- concentrations

between the large response lines versus small response lines. Further, there was no correlation between Na^+ and g_s ($r^2 = 0.09$, n.s.) or Cl^- and g_s ($r^2 = 0.10$, n.s.) for the 19 durum lines (Figure 5.5). From this it appears that Na^+ and Cl^- in the leaves were not responsible for genotypic differences in the response of stomatal conductance of lines grown in 150 mM NaCl.

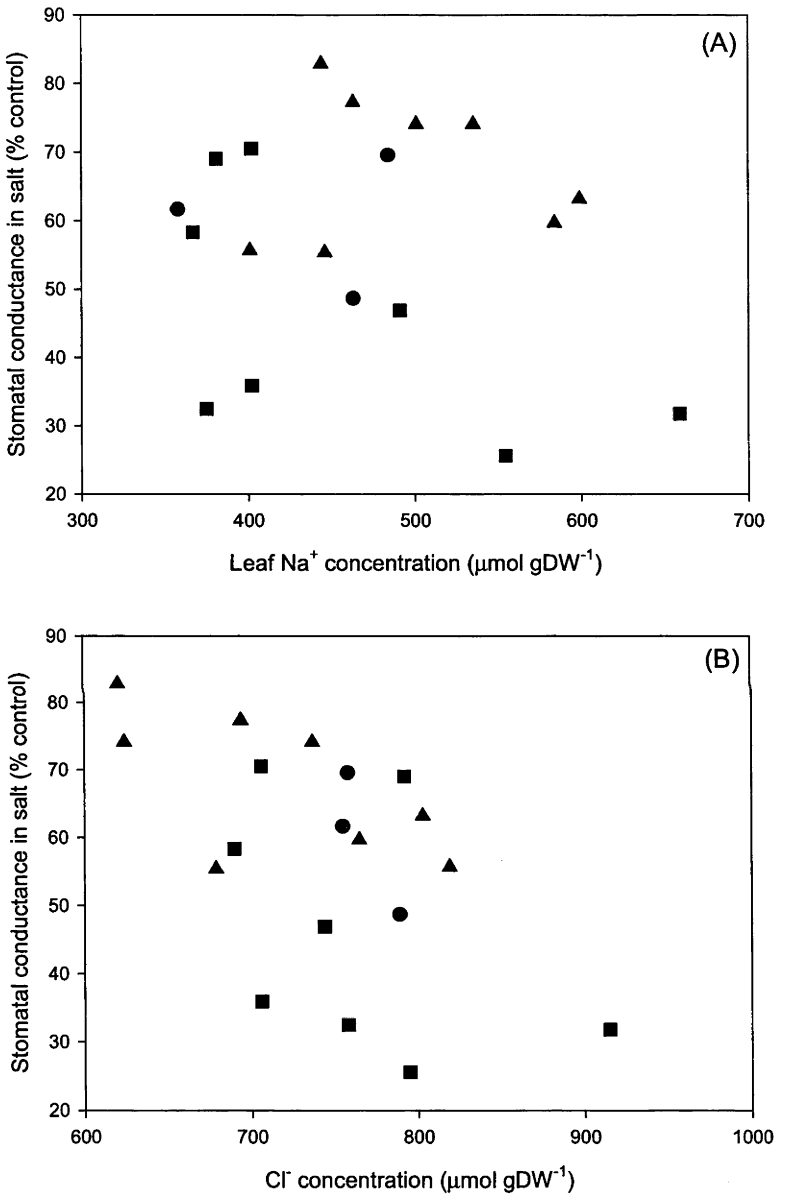

Figure 5.5 Relationship between (A) Na^+ concentration and (B) Cl^- concentration and stomatal conductance of salt-stressed durum cultivar checks (●), small response lines (▲) and large response lines (■). Points represent means (n=4).

Figure 5.6 shows the relationship between stomatal response of 19 durum lines from Experiments 1 – 3 and a repeat study (Experiment 4). The correlation co-efficient

derived from these data is analogous to an estimate of the repeatability of screening genotypic variation for stomatal conductance. An intermediate coefficient of determination of 0.43 and some re-ordering of line ranking across the experiments indicates that a large portion of the observed phenotypic variation for stomatal conductance was environmentally derived. Most of the rank change between sets of experiments was associated with a small number of the putative large response lines. Several lines selected originally as small response and large response lines maintained their classification in the repeat study.

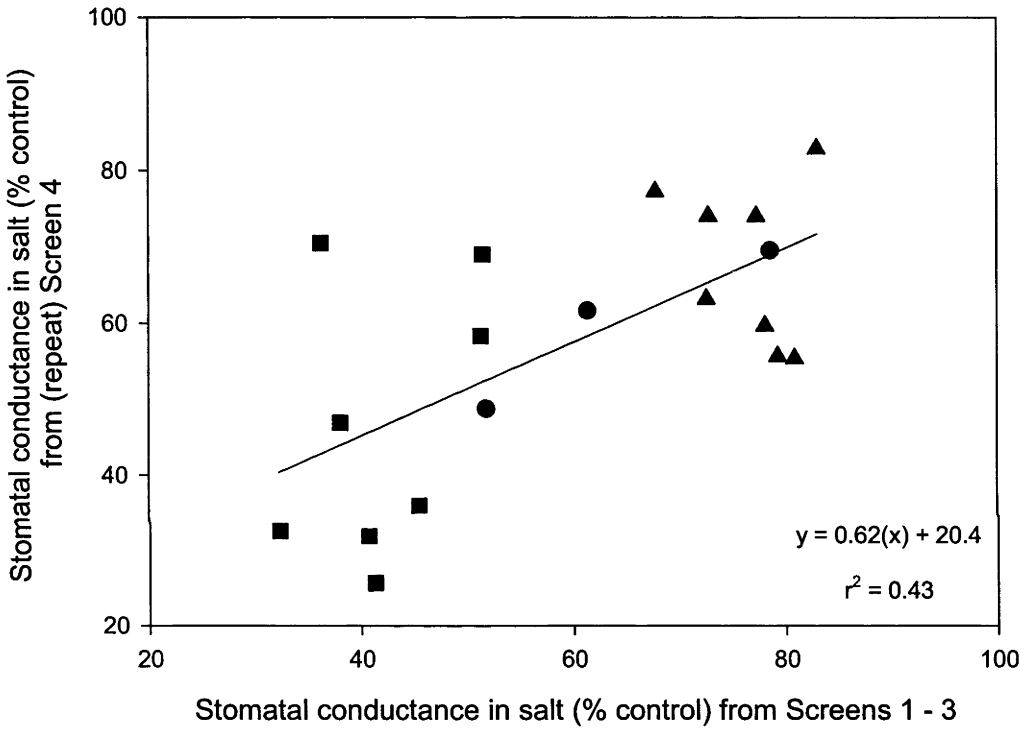

Figure 5.6 Relationship between stomatal conductance in salt (as a % of controls) in initial screening experiments (Screens 1 – 3) and repeat experiment (Screen 4) for checks (●), small response lines (▲) and large response lines (■). Points are means (n=4).

5.3.3 Relative growth rates and gas exchange measurements of a selection of small and large response lines (Experiment 5)

Three small response durum lines (Seklavi, Coulter, Hercules) and three large response durum lines (Durex, Candicans, Koelz W3158) were selected on the basis of a consistent stomatal conductance response in 150 mM NaCl (Experiment 4) and similar growth stage when the salt treatment commenced (Table 5.1). These lines together with current durum varieties Tamaroi and Wollaroi, provided the variation in g_s needed to

determine the impact of g_s on CO_2 assimilation rate (A) and also to examine the relationship between g_s and relative growth rate (RGR).

Stomatal conductance was reduced to a greater extent than A by the salt treatment (Table 5.2). While the average g_s was reduced by 50% due to salt, the average A remained unchanged in the low response lines. Similarly, a 70% decline in g_s due to salt was associated with only a 15% decline in A of the large response lines. This smaller decline of A than of g_s in the salt treatment relative to the control was unlikely to be due to increased photosynthetic capacity associated with stress-induced increased leaf thickness, as leaf 4 was fully expanded when the salt treatment commenced.

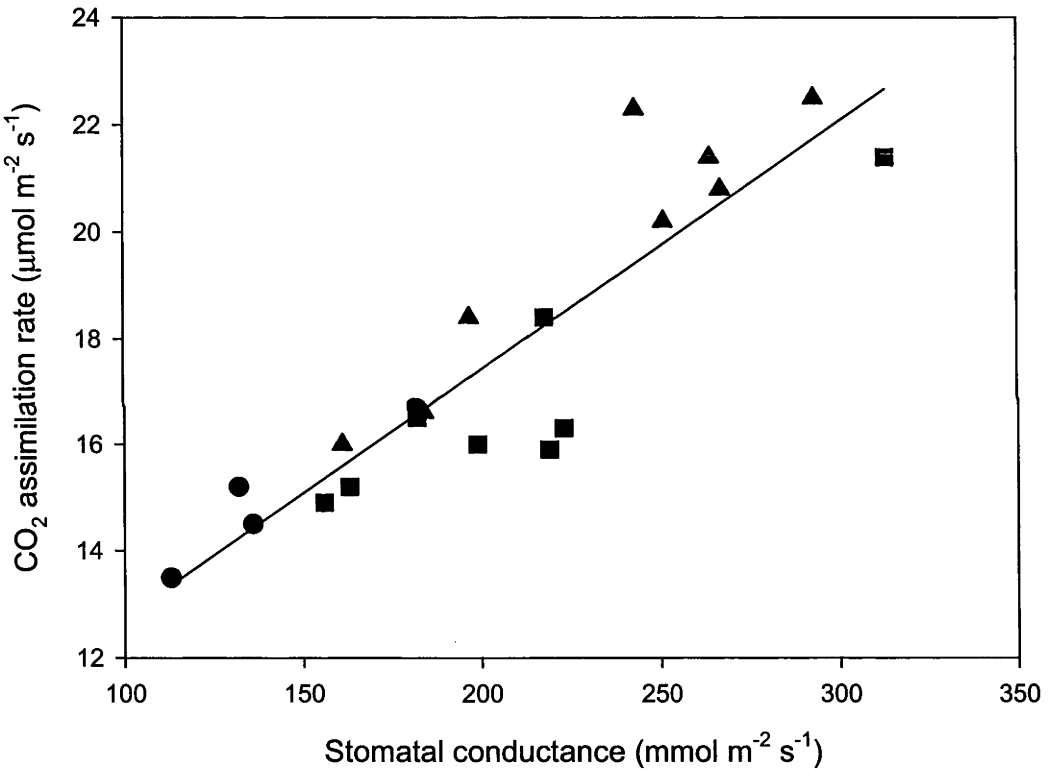

Figure 5.7 Relationship between stomatal conductance and CO_2 assimilation rate of small response lines (▲), large response lines (■) and durum cultivar checks (●), grown in 150 mM NaCl. All measurements taken on fully expanded leaf 4 of individual reps after 5 – 8 d in salt. Fitted linear regression is described by the following equation: $y = 0.047x + 8.1$ (r^2 = 0.82)

There was a strong correlation ($r^2 = 0.82$) between g_s and A amongst all lines grown in a high salinity treatment (Figure 5.7). A doubling in g_s from 150 to 300 mmol m^{-2} s^{-1} was associated with a 50% increase in A from 15 to 22 µmol m^{-2} s^{-1}. As these measurements were on leaves that were fully expanded before the salt treatment commenced,

Table 5.2 Stomatal conductance, CO_2 assimilation rate and relative growth rate of 3 small response durum lines, 3 large response durum lines and two current durum cultivar checks, Wollaroi and Tamaroi. Stomatal conductance was measured using an AP4 porometer, whereas CO_2 assimilation rate was measured using a LICOR 6400. Gas exchange measurements were taken from plants grown in either control (no salt) or 150 mM NaCl for 5 – 6 d (stomatal conductance) and 8 d (CO_2 assimilation rate). All values are means (n = 6 – 18) ± s.e.

Category	Stomatal conductance (mmol m^{-2} s^{-1})			CO_2 assimilation rate (µmol m^{-2} s^{-1})			Relative growth rate (g g^{-1} d^{-1})		
	Control	Salt	Salt (% control)	Control	Salt	Salt (% control)	Control	Salt	Salt (% control)
Small response lines (n = 3)	540 ± 14	255 ± 18	**47 ± 4**	20.0 ± 1.2	19.8 ± 0.9	**99 ± 4**	0.171 ± 0.006	0.143 ± 0.009	**84 ± 5**
Large response lines (n = 3)	524 ± 17	151 ± 20	**28 ± 3**	19.8 ± 0.8	16.8 ± 0.8	**85 ± 4**	0.178 ± 0.003	0.116 ± 0.007	**65 ± 4**
Checks (n = 2)	578 ± 10	211 ± 28	**37 ± 5**	21.5 ± 1.5	15.0 ± 0.7	**70 ± 3**	0.170 ± 0.003	0.134 ± 0.003	**79 ± 2**

and shortly after the salt treatment was imposed, and as Na^+ and Cl^- concentrations in leaf were relatively low (Table 5.1, Figure 5.5), it is likely that the reduction in A was due to stomatal effects alone. This was confirmed by a decrease in Ci with decreasing g_s (data not shown). Generally, the small response lines had higher g_s at 150 mM NaCl and consequently higher A than the large response lines or durum cultivar checks (Figure 5.7, Table 5.2).

Relative growth rates (RGR) did not differ greatly between lines in the control (non- saline) treatment, with most lines having a RGR between 0.170 – 0.185 g g^{-1} d^{-1} (Table 5.2). The exception was Hercules with a RGR of 0.153 g g^{-1} d^{-1}. However, the RGR of all lines apart from Seklavi decreased significantly due to salinity. The RGR of Seklavi in the salt treatment was 0.173 g g^{-1} d^{-1} (Figure 5.8) which was considerably higher than all other lines tested and appeared to be unchanged by the salt treatment (98.9 ± 5.8 % control). The largest reduction (35%) in RGR came from the large response lines, whereas RGR in the small response lines was reduced on average only

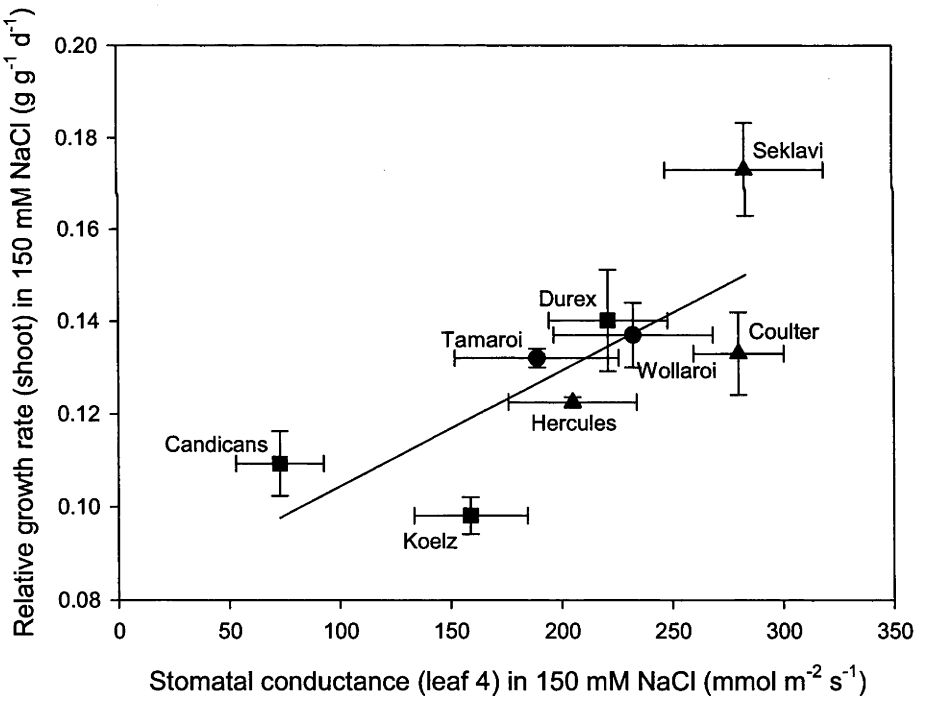

Figure 5.8 Relationship between stomatal conductance and relative growth rate of small response lines (▲), large response lines (■) and durum cultivar checks (●), grown in 150 mM NaCl. Relative growth rates of the shoot were determined over a 10 d period (2 – 12 d in salt) and stomatal conductance measurements were made on leaf 4 over a two d period (5 – 6 d in salt). Bars indicate S.E. Fitted linear regression is described by the following equation: $y = 2.45e^{-4}(x) + 0.08$ ($r^2 = 0.56$).

by about 15%. The RGR of the durum cultivar checks (Tamaroi and Wollaroi) was reduced by about 20% (Table 5.2).

Figure 5.8 shows the relationship between stomatal conductance of leaf 4 (5 – 6 d in salt) and the RGR of the shoot between 2 – 12 d in salt. There was a positive relationship between parameters (r^2 = 0.56). Lines with a higher g_s in 150 mM NaCl tended to have a higher RGR. Of the lines from the large stomatal response group, Durex had the highest RGR in salt of 0.14 g g^{-1} d^{-1}, which was similar to the mean RGR of the small stomatal response lines (Table 5.2). Seklavi was a standout line from the small stomatal response group, with the highest RGR and g_s in salt (Figure 5.8).

5.4 DISCUSSION

5.4.1 Genotypic variation for stomatal conductance in salinity

Genotypic variation in tolerance to osmotic stress was assessed by measuring g_s of plants grown in salt, relative to non-salt controls. The precision of this screening method depended on a number of key factors. Stomatal conductance measurements were taken on wheat leaves that were fully expanded prior to the commencement of the salt treatment. This avoided any potential genotypic variation in salt-induced changes to morphology during leaf development that may have influenced g_s, such as changes in stomatal density. Additionally, measurements were taken shortly after the final concentration of salinity was achieved (3 – 5 d), before salt levels could build up to potentially toxic concentrations of greater than 200 mM (James et al., 2002), and before gas exchange parameters might decline with leaf age (James et al., 2002). By applying this strategy, two to three fold differences in the stomatal response to salt-induced osmotic stress was found in a collection of 50 durum wheat genotypes (Figure 5.3, Table 5.1).

Jiang et al. (2006b) found that in barley, high g_s in salt was largely a function of high g_s in control conditions. This was not the case in this study of diverse durum genotypes. For the durum wheat grown in the present study, g_s under salt was independent of intrinsically high g_s. There was very little variation in the g_s of 16 durum selections under control conditions, but wide variation under salt (Figure 5.4).

Few studies have specifically set out to screen for g_s of salt-stressed plants, and when included in a set of potential tolerance parameters, gas exchange measurements

have typically been taken on leaves that had previously emerged in the presence of salinity (e.g. Isla et al., 1998; Ashraf and Shabaz, 2003; El Hendawy et al., 2005; Jiang et al., 2006b). Under such conditions, changes to a smaller leaf size may increase stomatal density per unit leaf area which may modify g_s. Additionally, some studies have reported g_s measurements that were extremely low even for control plants (e.g. ~100 mmol m^{-2} s^{-1}), indicating that the measurements were probably taken on old leaves, in low light, or at a very low relative humidity (e.g. Jiang et al., 2006b). Any genotypic variation in g_s is likely to be dampened under conditions such as these. Despite this, the above mentioned studies did demonstrate some genotypic variation in g_s of salt stressed wheat and barley.

The results from the current study have shown large genotypic variation in the response of g_s to salinity. The cultivar Seklavi and to a lesser extent cultivars Coulter and Hercules, showed the least stomatal closure in response to salinity and could be used as sources for osmotic stress tolerance in a breeding program. Little is known about Seklavi, other than that it is a landrace which originated from Turkey. Coulter and Hercules are Canadian varieties released for the eastern prairies in 1977 and 1969, respectively. It is very unlikely that any of these genotypes would be specifically adapted for Australian conditions; however, the trait identified in these genotypes, could be crossed into Australian durum cultivars and breeding lines using an appropriate phenotypic screen and also with the assistance of linked molecular markers. The decision to select for osmotic stress tolerance in a breeding program would be subject to validation of the contribution of this trait to salt tolerance based on grain yield under field conditions.

5.4.2 Relationships between stomatal conductance, CO_2 assimilation rate and relative growth rate

Higher g_s values in salt were related to higher A (Figure 5.7) and generally, a higher RGR (Figure 5.8, Table 5.2). This indicates the potential for using g_s to select for extremes in growth rate of salt-grown durum wheat genotypes. It was not clear from this study whether higher g_s and consequent higher A in the salt-stressed small response lines (Figure 5.7, Table 5.2) affected RGR, or whether selecting for high g_s was simply a surrogate for selecting genotypes with a higher RGR. It is possible for both of the above suggestions to occur concurrently or sequentially, if controlled by the same factor(s). Abscisic acid (ABA) is a likely candidate as it is known to control g_s (Davies

and Zhang, 1991) and increases in ABA have been correlated with inhibition of leaf elongation rate (He and Cramer, 1996; Montero et al., 1997; Cramer and Quarrie, 2002).

Growth studies on wheat and barely have shown that a decrease in A was largely responsible for a decrease in growth (RGR) of salinized plants. For example, in a comparative study using 13 wheat genotypes of contrasting salt tolerance, El-Hendawy et al. (2005b) concluded that a decline in photosynthesis was primarily responsible for the salinity-induced reduction in RGR in most genotypes. Similarly, Jiang et al. (2006b) also found significant variation in g_s among 14 barley genotypes in salt (20 dS m^{-1}) and concluded that g_s was the major limiting factor on both photosynthesis and growth (above ground biomass). In contrast, Isla et al. (1998) found no significant relationship between g_s and yield in a study using 34 barley genotypes grown at high salinity. However, the very low g_s measurements in that study (less than 50 mmol m^{-2} s^{-1}), perhaps as a result of a combination of a very high salinity (~23 dS m^{-1}) and also the time of the day of measurement, may have confounded this analysis.

Certainly in the longer term (weeks), maintaining higher A through higher g_s is essential for the maintenance of growth in saline conditions, as salt tolerance depends upon not only maintaining green leaf area with the potential for photosynthesis (through salt exclusion or efficient cellular partitioning), but also on maintaining leaf area with the sustained capacity to adequately supply carbon to roots and reproductive structures.

5.4.3 Possible problems with selecting lines with high stomatal conductance in salt

There are two potential negative consequences associated with the selection of lines with high g_s in saline conditions; compromised leaf water relations and increased salt build up in leaves.

Reductions in leaf turgor which could reduce growth rate are conceivable if the demand for water driven by high stomatal conductance is not met by supply from the roots in a saline soil. Despite the fact that water relations measurements were not included in the present study, there was no evidence of wilting (reduced turgor) in any of the lines tested and furthermore, we found a positive relationship between g_s and RGR, which indicates indirectly, that turgor was not compromised in lines with high g_s in salt.

These observations appear to be consistent with similar studies reported in the literature. For example, El-Hendawy et al. (2005b) found that while there was significant genotypic variation in g_s of 13 wheat genotypes differing in salt tolerance when grown in a range of salinities, there were no significant genotypic differences in turgor. In that study turgor either remained the same as in the control treatment or increased with salinity. Others studies have also found no significant correlation between g_s and turgor in salt-stressed wheat (Ashraf and Shahbaz, 2003; Rivelli et al., 2002).

Another possible repercussion of selecting wheat genotypes with higher g_s (and therefore higher transpiration rates) under saline conditions, is that salts could build up to higher concentrations in the leaves. In wheat however, shoot ion uptake is not determined by transpiration rate, as the processes which regulate water uptake and ion uptake are largely independent (Munns, 1985; Ball, 1988; Munns et al., 2006). This was also confirmed by results from the present study, where there was no correlation between Na^+ or Cl^- concentration and stomatal conductance (Figure 5.5). Rice is an exception to this, as 'transpirational bypass flow' contributes substantially to the uptake of Na^+ into the shoot (Yadav et al., 1996; Garcia et al., 1997). For example, transpirational bypass flow was an order of magnitude higher in rice line IR36 than wheat landrace Kharchia, resulting in a doubling of Na^+ concentration in the xylem of rice (Garcia et al., 1997).

5.4.4 Screening for stomatal conductance

As screening techniques need to be simple, relatively cheap, quick, reliable and repeatable, there is an apparent compromise between precision and speed (frequency) when screening for g_s. Reliable g_s measurements could be taken using a closed gas exchange system (e.g. LICOR 6400) where light and VPD can be tightly controlled to imitate growth conditions. However, these measurements typically take 5 – 10 min to complete per leaf and therefore the accuracy of measurement is at the expense of the number of measurements possible in a given time frame. Faster measurements of g_s taking less than 10 s per leaf are possible when using a cycling diffusive porometer as used in this study, or a viscous flow porometer (Rebetzke et al., 2000), but need to be repeated across reps, days and preferably environments to more accurately characterize a phenotype. Other techniques to estimate g_s (e.g. thermocouples, infrared thermometer) were evaluated but not reported here, as they were considered impractical.

Rapid cycling temperature fluctuations in the controlled environment chambers, together with the irregular orientation of wheat leaves were major complicating factors which precluded using leaf temperature-dependent techniques to estimate g_s. The viscous flow porometer has been used with some success when measuring g_s of flag leaves in field grown wheat plants (e.g. Rebetzke et al., 2003). This instrument however, proved unreliable in this study (other than for extremes in g_s) due to differences in leaf thickness (SLA) and damage to leaves because of the relative 'fleshiness' of young (leaf 4) growth chamber-grown wheat leaves. For example, SLA ranged between 243 – 311 among 20 genotypes in Experiment 1 and FW:DW ratio of leaf 4 for most genotypes was about 6 (cf. 3 – 4 of flag leaves from field grown plants).

Finally, g_s measurement of salt-stressed durum wheat should ideally be validated in the field. These measurements could be problematic due the large environmental variation both below (heterogeneous salinity) and above ground (wind, temperature, relative humidity and light) that would affect g_s and be difficult to account for.

Selection for g_s may be difficult, as g_s is probably controlled by a number of genes (Rebetzke et al., 2001; 2003) but principally because environmental effects are likely to be large (Richards et al., 2001; Rebetzke et al., 2003). An estimate of repeatability for screening genotypic variation in g_s in 19 durum lines common to Experiments 1 – 3 and Experiment 4 was only moderate (Figure 5.6). This may be due in part to the fact that this estimate incorporated 4 g_s measurements (2 control and 2 salt treatments) across sets of experiments. Not all genotypes retained their classification across experiments. This result indicates the importance of repeating and therefore validating g_s measurements. In a controlled environment (growth) cabinet, variation in some environmental factors that may affect g_s are reduced, but there is still variation in light intensity (spatial) and relative humidity which affect g_s measurements. Additionally, other studies point to daily and time of day effects on g_s (Roark and Quisenberry, 1977, Radin et al., 1994; Rebetzke et al., 2003).

5.4.5 Summary and conclusions

In summary, two to three fold differences in the response of g_s to salt-induced osmotic stress was found in a collection of 50 durum wheat genotypes. Higher g_s values in salt were related to higher A and there was a positive relationship between g_s and RGR in salt. The results described in this chapter are essentially a 'snap-shot' in time, which

has captured the potential for genetic gains to be made in tolerance to the osmotic stress in durum wheat. Further experiments are required to determine whether the potential is actually realized in terms of long-term growth and yield.

Chapter 6

GENERAL DISCUSSION

6. GENERAL DISCUSSION

6.1 Model of growth in response to salinity

Understanding the physiological framework for how the growth of plants is limited in saline soils is imperative to recognizing possible tolerance mechanisms and their potential impact on improving plant growth and yield. To this end, Munns (1993) proposed a two-phase model to describe the growth of plants in response to salinity. In summary, the initial decrease in growth is due to the osmotic stress of salinity – a decrease in the soil water potential. This affects the rate of leaf growth and tiller production for a wheat or barley plant in much the same way as drought. Growth is then further decreased with time due to a reduced supply of assimilate to growing tissues. This is a result of leaf death due to salts building up to toxic levels in the older mature leaves.

The key implication of this growth model is that differences in salt tolerance would appear over time (weeks) and be related to differences in leaf injury. Therefore to a large degree and justifiably so, much research has focused on understanding the physiology of tolerance mechanisms which reduce salt-induced leaf injury; primarily Na^+ exclusion (e.g. Läuchli, 1984; Munns, 1993; Tester and Davenport, 2003) but also tissue tolerance (e.g. Greenway and Munns, 1980; Yeo and Flowers, 1983; James et al., 2002), and searching for genotypic variation in these salt tolerance traits (e.g. Yeo et al., 1990; Schatchman et al., 1991; Munns et al., 2000b; Munns and James, 2003).

However, the results from gas exchange studies in Chapters 3 and 5 have now highlighted the impact and relative importance of stomatal limitations on CO_2 assimilation rate and growth of salt-stressed durum wheat. In the study with Wollaroi and Line 255, two genotypes with contrasting leaf injury in salt, stomatal conductance was reduced early in the life of the leaf and was the initial cause of reduced CO_2 assimilation rate. This limitation continued over the course of the stress. Eventually, additional constraints on CO_2 assimilation occurred in the form of non-stomatal limitations, as Na^+ increased to toxic concentrations in the leaf and directly affected photosynthetic biochemistry. Additionally, there was a positive linear relationship between stomatal conductance and CO_2 assimilation rate of durum genotypes with differences in stomatal response when grown in a high salinity, before salts built up to high concentrations in the leaf tissue (Figure 5.7). These data collectively indicate that

the stomatal limitation on photosynthesis is a major factor limiting growth of durum wheat in salinity. Others have made similar conclusions for barley (e.g. Chen et al., 2005; Jiang et al., 2006b) and wheat, (e.g. El Hendawy et al., 2005) and also for other species such as olive (e.g. Loreto et al., 2003; Centritto et al., 2003). It is likely that these stomatal limitations are not brought on by salt-specific stress (ion toxicity), but rather by the osmotic stress component of salt stress.

Figure 6.1 is a schematic diagram which summarizes and integrates some of the findings of this thesis into the previous model of growth response to salinity proposed by Munns (1993). Together with the salt-specific affect which impacts on growth in the long term and the immediate effect of osmotic stress on leaf elongation rate, Figure 6.1 incorporates an additional immediate effect of osmotic stress on reduced stomatal

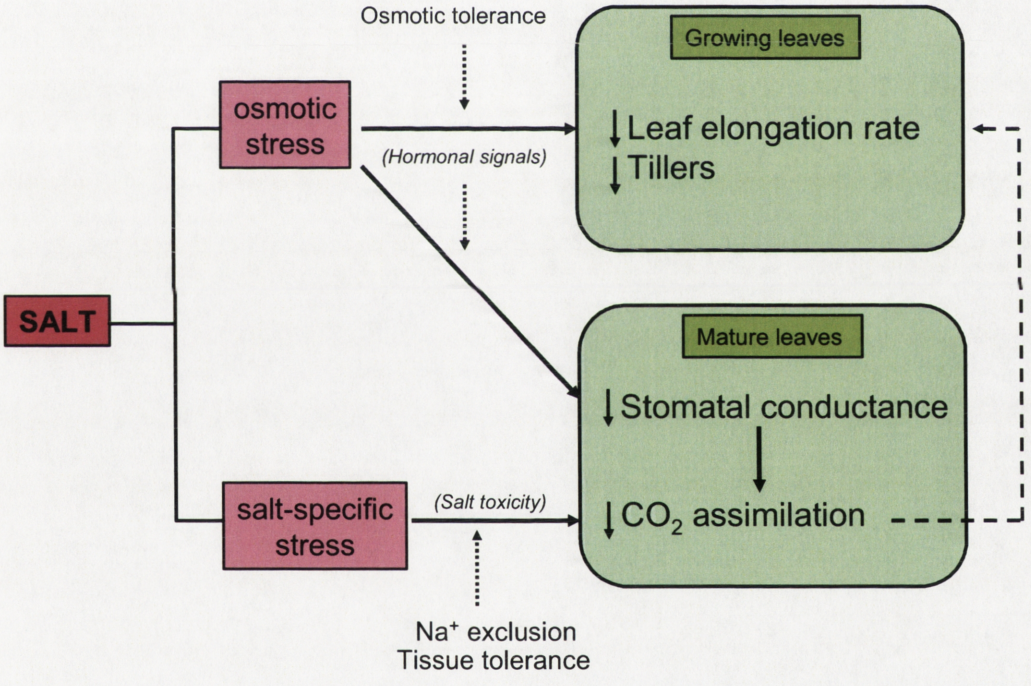

Figure 6.1 Model for plant growth in response to salinity.

conductance, and a resultant reduction of CO_2 assimilation. This stomatal response is possibly affecting growth in the short term (days to weeks) through reduced C supply, as well as in the long term (weeks to months) when leaves begin to die. Previously, it was assumed that tolerance mechanisms only related to reducing the salt-specific affect on leaf injury (Na^+ exclusion and tissue tolerance). However, the results in Chapter 5,

showing genotypic variation in stomatal response, suggest that there is also potential for sources of tolerance to osmotic stress.

It is possible that in the long term, higher stomatal conductance and subsequent higher CO_2 assimilation rates under salinity may lead to higher yields through greater number of tillers. Nicolas et al. (1993) concluded that tillering in wheat was greatly reduced by salinity, but that high CO_2 partly reversed this effect. A similar relationship between greater numbers of tillers and elevated CO_2 has also been observed in salt-affected rice (Wilson et al., 2005). One explanation for these observations is that under salinity, low concentrations of key carbohydrate metabolites or soluble carbohydrates may be limiting tiller formation (Nicolas et al., 1993). This may have been partly ameliorated by elevated CO_2 through increased production of these compounds

In summary, salt tolerance therefore depends upon mechanisms for maintaining productive green leaf area, not only in terms of potential (total photosynthesising leaf area), but also in terms of activity (rate of photosynthesis per unit leaf area). Both are important for maintaining an adequate supply of carbon to growing tissues and reproductive structures. For crop plants such as wheat and barley, this should result in the initiation and growth of tillers, the establishment of fertile florets and the subsequent supply of assimilate to fill ears.

6.2 Application of osmotic stress tolerant and tissue tolerant genotypes in the field

In this thesis the possibility of screening for osmotic stress tolerance was explored. The hypothesis was that osmotic stress tolerant lines would have a 'small stomatal response' to salinity, i.e. would maintain a higher stomatal conductance under salt stress, leading to higher CO_2 assimilation and growth. An important question remains. How would lines with this trait perform in the field?

The efficacy of selecting for genotypes with osmotic stress tolerance will depend on climatic conditions and type of salinity. Osmotic stress tolerance may be an advantage for crops grown in soils with transient salinity and a winter dominant rainfall pattern (southern and western Australia) or where rainfall is more evenly distributed (southern NSW and Victoria). Under these conditions, the salts may not concentrate to a large extent in the rhizosphere until later in the season and therefore high stomatal conductance may lead to better use of available water and higher yields.

In regions with a summer dominant rainfall pattern (northern NSW and Queensland), a more conservative water use approach may be appropriate. In these areas, where the crop is surviving longer on stored water, water availability decreases throughout the season and the salt concentration in the soil steadily rises. It may be appropriate to grow varieties with conservative water use traits such as low stomatal conductance and high transpiration efficiency (Condon et al., 2002). This may reduce the rate at water is consumed in the subsoil, thereby reducing the rate at which salts concentrate around the roots and therefore leaving sufficient water in the soil to fill grain later in the season.

It is envisaged that tissue tolerance may have a more universal application across salinities of varying severity, but may have greater impact at higher salinities, particularly towards the end of the growing season as salts concentrate in soils with transient salinity. Na^+ exclusion in durum wheat was found to have a beneficial effect on yield at moderate salinities, but not at high salinities (Husain et al., 2003). These authors concluded that pyramiding traits such as tissue tolerance with Na^+ exclusion should result in greater salt tolerance. However, it is possible that effective compartmentation of salt in leaf cells (tissue tolerance) and associated osmotic adjustment is a more effective salt tolerance trait than Na^+ exclusion at high salinities, where leaf turgor maintenance is more vulnerable.

6.3 Screening for salt tolerance

6.3.1 Screening strategies

Traditional screening techniques have used agronomic selection criteria such as biomass accumulation and yield to examine genotypic differences in salt tolerance. However, there are problems with using field based approaches in salt tolerance screens due to the heterogeneity of saline fields and also differential growth and developmental patterns between genotypes and species. There is also logistical, time and cost considerations involved in long term growth studies.

A trait-based physiological screening approach was used in this thesis in order to reliably and quickly assess genotypic variation in salt tolerance traits in large germplasm collections. But this is just the first step. Once lines with desirable traits are identified and the traits incorporated into appropriate adapted backgrounds, yield evaluation of new lines on well characterized saline field sites is essential for validating

the relative impact of the traits. If the heterogeneity of saline field sites was very high, biomass and yield assessment could be examined in a pot trial in a controlled environment using soil collected from saline field sites.

6.3.2 Screening germplasm collections for salt tolerance traits in related species

In this thesis, a collection of about 50 durum and durum-related landraces, with representatives from each of the five *T. turgidum* subspecies, was screened for variation in tissue tolerance (Chapter 2). A second screen for osmotic stress tolerance also included a diverse range of durum varieties from around the world (Chapter 5). Both of these investigations found genotypic variation in salt tolerance traits with potential to improve yield in saline soil.

There are good prospects for discovering genetic variation for salt tolerance in wheat and barley using old varieties (landraces) and progenitors and related species (reviewed by Colmer et al., 2005). Kharchia 65 is a well-known example of an Indian wheat landrace that was identified with high salt tolerance (Joshi, 1976; Kingsbury and Epstein, 1984). Significant variation for Na^+ exclusion has been identified in *T. tauschii* accessions (Schachtman et al., 1991) and in K^+/Na^+ discrimination in synthetic hexaploids derived from *T. monococcum* and *T. boeticum* (Gorham, 1990). Recently, novel genes for Na^+ exclusion in a durum landrace (Line 149), *Nax1* and *Nax2*, were also found to have originated from a *T. monococcum* accession (James et al., 2006).

There may be sufficient variation within crop species including landraces, for tolerance to moderate salinity, but not severe salinity. Novel sources of tolerance to soils with higher concentrations of salt are likely to exist in wild relatives of wheat and barley (Colmer et al., 2005; 2006). There may be a trade-off however, in gains in tolerance made from wild relatives and progenitors of wheat, because of possible yield penalties associated with linkage drag when crossed into modern high yield-potential cultivars. International durum wheat varieties and landraces are more likely to have good expression of most agronomically important characters. Therefore, there are inherent safeguards in screening firstly within this gene pool and advantages in crossing and germplasm development using these compared to wild relatives of durum wheat (i.e. *T. dicoccoides*). The most important safeguard concerns screening for traits that may be confounded by other factors such as genotypic variation in vigour, plant height, flowering and maturity time (Yeo et al., 1990). There are advantages in the potential for

selection of new commercially adapted breeding lines from just one cross of a cultivar or landrace with a local adapted variety. At a practical level, there is also likely to be better synchrony in flowering and relative ease of pollination.

6.3.3 Alternative phenotypic screens for tissue tolerance and osmotic stress tolerance

The relationship between Na^+ content and leaf injury was used as a screen for tissue tolerance to high leaf Na^+ concentrations (Chapter 2). This screen proved to be useful as an initial 'coarse' filter, particularly in differentiating between extremes. However, it cannot accurately account for location of ions in the leaf, a character shown to be important for salt tolerance in barley (Chapter 4), nor the subtle changes in photochemistry associated with Na^+ accumulating to about 250 mM (Chapters 3 and 4), which marks the onset of non-stomatal limitations on photosynthesis.

An improved physiological screen for tissue tolerance would ideally incorporate a range of non-destructive measurements to assess parameters including photochemistry, chlorophyll and other plant pigments, water status, gas exchange and ion concentrations or fluxes. There are a number of techniques that can be used and probably should be used in combination. These include chlorophyll fluorescence, spectral reflectance, SPAD measurements (chlorophyll content), porometry and thermal imaging. Spectral reflectance in particular, has the potential to estimate a number of important parameters simultaneously. It has been used successfully in the field at the canopy level to predict yield and water status of irrigated bread wheat and durum wheat (Babar et al., 2006; Royo et al., 2003), and barley grown in saline soils (Peñuelas et al., 1997). This technique could be adapted for use on single plants. Indices could be developed for water status, plant pigments indicative of photochemical 'stress' (xanophylls and carotenoids) and photosynthetic capacity (N and chlorophyll) (Sims and Gamon, 2002).

Monitoring leaf water relations will be important in lines with small stomatal response to osmotic stress (higher stomatal conductance in salt) as they may be more susceptible to loss of turgor. Relative water content (RWC) has been used to monitor leaf water deficit in many drought and salinity studies; however, recent research has shown that for salt stressed wheat and barley, RWC bore little relationship to leaf turgor (J Boyer, unpublished results). A screen for osmotic stress tolerance would therefore need to include a more reliable measure of leaf water status than RWC. As indicated

above, it is possible that water indices of spectral reflectance, both in the visible and higher wavelengths, may be developed (ground-proofed) to give a more dependable and non-destructive estimate of leaf water status.

6.4 Possible genes and transporters involved in tissue tolerance and osmotic stress tolerance

6.4.1 Tissue tolerance

Tissue tolerance is a function of a number of coordinated processes which maintains the Na^+ concentration in the cytoplasm at low and non-toxic concentrations. The compartmentation of Na^+ into the vacuole is integral to this process. If Na^+ (and Cl^-) are sequestered in the vacuole of the cell, K^+ and organic solutes need to accumulate in the cytoplasm and organelles to balance the osmotic pressure of the ions in the vacuole.

Efficient cellular and sub-cellular partitioning of Na^+ and K^+ in barley cultivar Franklin led to the preservation of a favourable K^+:Na^+ ratio in the cytoplasm of mesophyll cells at higher leaf Na^+ concentrations in comparison with durum wheat Wollaroi. As a result, photosynthetic capacity was maintained at higher leaf Na^+ concentrations in the more tolerant barley compared to the sensitive durum wheat (Chapter 4).

At the sub-cellular level, the capacity to compartmentalise salts in the vacuoles, and thereby keep cytoplasmic concentrations low, is likely to be controlled by a vacuolar Na^+/H^+ antiporter (*NHX1*). *NHX1* transports Na^+ from the cytoplasm into the vacuole, a process driven by an electrochemical gradient across the tonoplast (Barkla et al., 1995; 2002; Blumwald, 2000). Overexpression of the vacuolar Na^+/H^+ antiporter in Arabidopsis (*AtNHX1)* resulted in an increase in salt tolerance (Apse et al., 1999) and also in tomato and Brassica (Zhang and Blumwald, 2001; Aharon et al., 2003). Similarly, transgenic wheat expressing the vacuolar Na^+/H^+ anitporter gene from arabidopsis (*AtNHX1),* showed improved growth and higher grain yield (compared to wild type) when grown in moderate salinities (Xue et al., 2004). However, the main benefit of this transformation may have been reduced transport of Na^+ to the shoot, as shoot Na^+ concentrations were significantly reduced in the transgenic lines grown in 150 mM NaCl.

Another process involved in increasing Na^+ uptake into the vacuole is increasing the activity of the H^+-pump on the tonoplast, thus increasing the electrochemical

gradient which energizes Na^+/H^+ antiporters. Some evidence for this came with the overexpression of the vacuolar H^+-pyrophosphatase gene (*AVP1*) in Arabidopsis, which was claimed to increase salt tolerance, although no quantitative growth data was presented (Gaxiola et al., 2001).

The maintenance of photosynthetic capacity at high leaf Na^+ concentrations in barley was related to a favorable K^+:Na^+ ratio (greater than 1.0) in the cytoplasm (Chapter 4). A similar result was achieved by transforming cotton with the vacuolar Na^+/H^+ antiporter from Arabidopsis (He et al., 2005). The *AtNHX1* transgene, gave a 20% increase in CO_2 assimilation rate of cotton grown in 200 mM NaCl for 28 d, compared to wild type. Similar increases were reported for both growth (shoot FW) and (fibre) yield. Importantly, there were no differences in photosynthesis between the transgenic and wild type cotton under control (no salt) conditions. Comparable increases in photosynthesis were also found by expressing the *Suaeda salsa* Na^+/H^+ anitporter (*SsNHX1*) in transgenic rice (Zhao et al., 2006).

The factors regulating cellular partitioning of ions in the leaf are not well known but probably are of equal importance. Of particular interest are the processes which regulate differential K^+ partitioning. A greater proportion of K^+ was partitioned to mesophyll cells compared to epidermal cells in the barley compared to the durum wheat, and this probably had a profound effect on the difference in the cytoplasmic K^+:Na^+ ratio between the two species (Figure 4.4). Some likely candidates for regulating cellular K^+ partitioning in the leaves are the high affinity K^+ tansporters, HKTs and HAKs (Horie et al., 2001; Su et al., 2002; Rodríguez-Navarro and Rubio, 2006). There is also evidence to suggest a role for K^+ selective inward rectifier channels KIRs (summarised by Shabala, 2003), although their role may be one of osmoregulation, rather than K^+ partitioning *per se* (Shabala et al., 2000).

One line of evidence for the HKTs comes from a recent study on rice which utilised both tissue expression analysis (using RT-PCR) and cell-specific expression analysis (through *in situ* PCR) of *OsHKT1* and *OsHKT2* (Kader et al., 2006). There was a 15 fold induction of *OsHKT2* in the leaves of salt-tolerant variety Pokkali grown in 150 mM NaCl, compared to plants grown in non-saline conditions, and this was about 10 fold higher than for the salt-sensitive variety BRRI Dhan29. Also, the expression of *OsHKT2* was largely confined to the phloem, to cells connecting the phloem to mesophyll cells and to mesophyll cells. From these results the authors

suggested that *OsHKT2* was involved in controlling Na^+/K^+ homeostasis by increasing K^+ uptake and recycling in leaf tissue. While the results of this study potentially support observations for cellular K^+ partitioning, it must be noted that other candidate genes, including more from the HKT family, were not incorporated in the study. Cellular partitioning of salt ions is likely to involve the coordinated activity of a number of transporters. Interestingly and perhaps somewhat counter-intuitively, the salt-tolerant variety used in the study (cv. Pokkali) is thought to be salt tolerant because of low rates of Na^+ transport to the shoot, not because of tolerance of high Na^+ levels in leaf tissue (Yeo et al., 1990).

6.4.2 Tolerance to osmotic stress

Stomatal conductance and leaf growth rate were reduced by the osmotic stress from salinity (Chapters 2 and 5). One likely candidate for regulating these processes is ABA, as it is known to control stomatal conductance (Davies and Zhang, 1991) and in many studies has been correlated with reduced leaf growth under saline conditions (e.g. He and Cramer, 1996; Montero et al., 1997). Other studies have concluded that a differential sensitivity to ABA was responsible to variation in a stomatal response to osmotic (salt) stress (Cramer and Quarrie, 2002). Additionally, as ABA is thought to act as a regulator of tissue water transport through water channel activity (Hose et al., 2000), its possible role as a growth regulator under water limiting conditions is further enhanced (Fricke et al., 2006).

Candidate genes responsible for regulating stomatal conductance and possibly leaf extension of osmotically (salt) stressed plants are likely to be genes related to ABA biosynthesis and also genes regulated by ABA. An example of a gene controlling the production of ABA under stress is 9-cis-epoxycarotenoid dioxygenase (NCED). Stomatal conductance of transgenic tomato overexpressing NCED (*LeNCED1*) was reduced by about 50%, which coincided with a 3 fold increase in leaf ABA (Thompson et al., 2000). Other studies with grapevine have shown similar correlations between increased expression of NCED (*VvNCED1*), increased leaf ABA and decreased stomatal conductance (Soar et al., 2004).

ABA can regulate leaf cell expansion through various signal transduction pathways responsible for controlling the uptake of solutes important for growth. For example, ABA has been shown to upregulate the transcription of an arabidopsis Na^+/H^+ antiporter *AtNHX1* (Shi and Zhu, 2002), and is most likely responsible for the

upregulation of other ion transporter genes. Another class of osmotic stress proteins regulated by ABA are the late-embryogenesis abundant (LEA) proteins (summarised in Chinnusamy et al., 2005). The LEA-type proteins appear to impart a dehydration protective effect at a cellular level, and therefore may be important in maintaining the integrity or turgor of expanding and elongating cells.

6.4.3 Development of molecular screens

Yeo et al., (1990) argue that one of the limitations of using physiological traits in plant breeding is that often many individuals are needed to obtain a single assessment of a genotype's phenotype. This therefore limits early generation selection unless combined with the development of double haploid (fixed) lines. The development of molecular markers may reduce the time, work and cost involved in trait-based phenotypic screens, although often, markers have been found to be cross-specific which limits their utility.

To identify a molecular marker, a specific highly repeatable phenotype developed from a physiologically-based screening technique is required, followed by population development, bulk segregant analysis and population genotyping for QTL analysis. Once a locus (QTL) that accounts for a significant proportion of phenotypic variation of a specific trait is identified, a PCR-based molecular marker can be developed. Markers can be tested on seeds or seedlings, and provide an efficient way of screening large numbers of individuals in an early generation (F_2) segregating population (see below).

Molecular markers appear to represent the ultimate in a selection technique, as their use is non-destructive, and does not require controls or salt treatments. However, a simple phenotypic screen for a complex trait that is highly heritable can be more efficient than the use of molecular markers, as it avoids the time and costs involved in the initial identification and development of the markers.

A second approach to the development of molecular markers may be to search for differential expression of a candidate gene known or thought to be involved in tissue tolerance and/or osmotic stress tolerance. An example of this was discussed previously (Section 6.4.1), where differential expression of OsHKT2 was found in rice genotypes of contrasting salt tolerance (Kader et al., 2006). As there is an orthologue of the rice HKT2 in durum wheat (S Huang, unpublished results), expression analysis using RT-

PCR could be used to indicate function, with the view of then developing PCR based molecular markers for use in durum wheat.

6.5 Breeding strategies for improving salt tolerance of durum wheat.

Salt tolerance is a complex trait. Occasionally, the introduction of a single gene from a salt tolerant source can have profound effects (e.g. James et al., 2006), but this is usually not the case (Flowers and Yeo, 1995; Flowers, 2004). It is likely that both tissue tolerance and osmotic stress tolerance involve the interaction of a number of physiological and biochemical process, and therefore are probably regulated by a number of genes. This will have implications for the type of breeding strategy used to incorporate these traits into adapted varieties.

Large populations need to be developed to be able to select for the unique individuals containing all the genes of interest; salt tolerance genes from the donor parent and important agronomic characters from the recurrent 'adapted' parent. For example, the population size needed to recover at least one individual fixed at 4 loci at a 95% probability of success is 766. For 5 loci, the size of the population increases to 3067. Various strategies that can be used to reduce population size and facilitate a more manageable screening process include inbreeding, development of double-haploid populations, backcrossing to increase the frequency of recurrent parent alleles, and F_2 enrichment using molecular markers for selection of individuals that are carriers of target alleles (Bonnett et al., 2005).

The recurrent parent should be the highest yielding best locally adapted durum variety for a given location. In Australia, the best locally adapted durum varieties in northern NSW (cv. Bellario) are different from those that perform best in South Australia (e.g. cv. Tamaroi). Precedent for this strategy comes from Richards (1983; 1992) who argued that the best breeding strategy for salt tolerance is to select the highest yielding lines under non-saline conditions (i.e. breeding for yield potential).

Can greater advances in salt tolerance be made by combining or pyramiding individual salt tolerance traits (Yeo and Flowers, 1986; Noble and Rogers, 1992)? Important physiological questions need to be addressed first. For example, what are the physiological ramifications of combining tissue tolerance with Na^+ exclusion? Barley, a tissue tolerant ideotype, appears to effectively use salt ions as a 'cheap' source of osmotica, for osmotic adjustment and turgor maintenance. If salt exclusion were to be

combined with a tissue tolerant barley or durum wheat, would there be sufficient K^+ or other inorganic osmotica to cheaply make up for the decrease in Na^+, or would there be a need for the more costly synthesis of organic solutes?

6.6 Future research

6.6.1 Osmotic stress tolerance

It was concluded at the end of the study into tolerance to osmotic stress that further investigations would be required to determine whether the potential observed in some lines with only a small stomatal decline in response to high salinity eventually translates to improved long-term growth and yield, in comparison to lines where the stomatal response to salinity is large. This would require longer term gas exchange measurements to determine if the small g_s response and subsequent higher A in salinity is maintained over a longer time period (weeks to months) and next, field studies to see if this translates to higher grain yields.

6.6.2 Tissue tolerance

Reliable measurements of cytoplasmic ion concentrations in mesophyll cells are needed to accurately characterize tissue tolerance. In this thesis, cytoplasmic concentrations were calculated from vacuolar concentrations (using cryo SEM X-ray microanalysis), whole tissue concentrations and aqueous volume proportions of cell types. This approach was very detailed and time consuming and not without sources of error, although the errors were systematic and applied to the calculation of all ions. K^+ and Na^+ selective microelectrodes have been used to measure cytosolic activities in roots (Walker et al., 1996; Carden et al., 2003) and K^+ activities in the leaves of salt-stressed barley (Cuin et al., 2003). To date, cytosolic Na^+ activities have not been measured in leaves using this technique.

Another approach worth more investigation is the use of specific ion indicators or dyes. Na^+ binding fluorescent indicators such as SBFI have been used for subcellular measurement of Na^+ (Mühling and Läuchli, 2002b; Halperin and Lynch, 2003). However there are concerns with the specificity of such indicators. Recently a new sodium indicator dye CoroNa Green has become available which is potentially more sensitive and specific for Na^+ than earlier compounds. Development of an appropriate protocol for the use of this dye, coupled with the use of a confocal laser scanning

system may allow for semi-quantitative measurements of subcellular Na^+ concentrations.

6.6.3 Factors controlling leaf ion homeostasis

In Chapter 3, two tetraploid genotypes exhibited tight control of Na^+ accumulation in leaf 3, over a period of 7 – 14 d, before Na^+ eventually began to rise to concentrations that coincided with leaf injury and therefore were considered toxic (Figure 3.4). A similar profile of Na^+ homeostasis was evident among 4 wheat lines with different Na^+ exclusion capabilities (Rivelli et al., 2002).

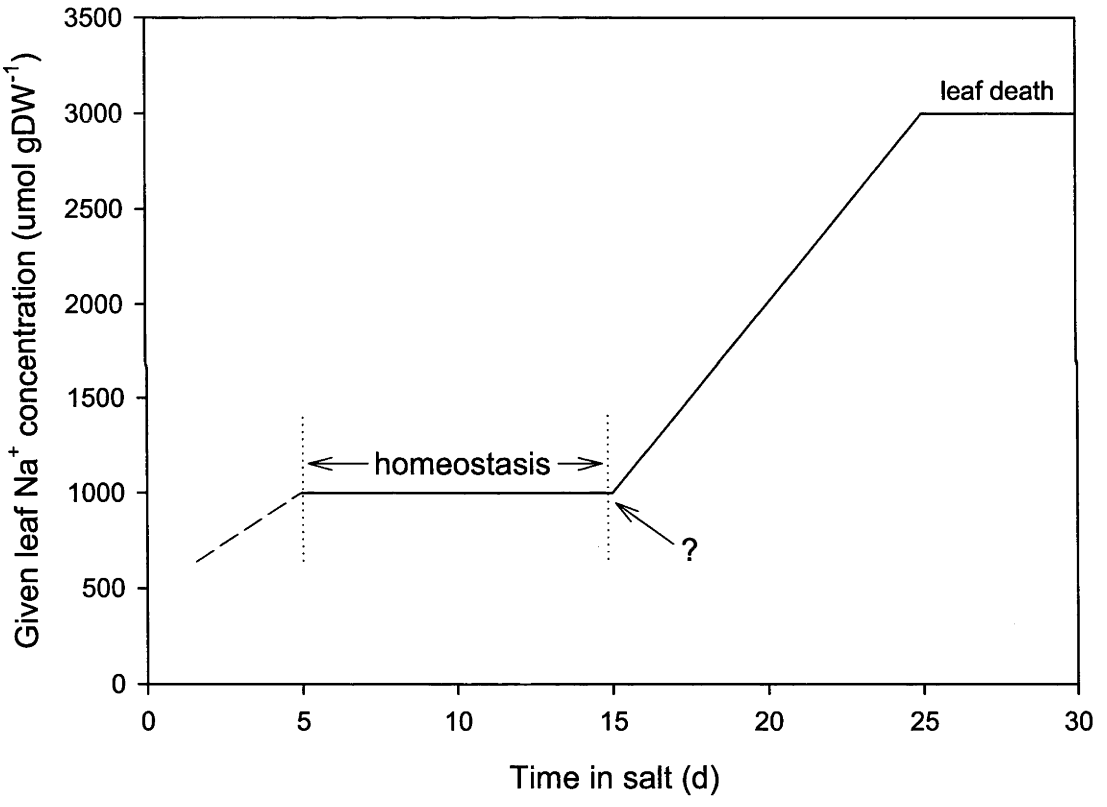

Figure 6.2 Schematic of a given leaf Na^+ accumulation profile of a salt-stressed wheat or barley plant

An important question that requires examination relates to what factors control Na^+ homeostasis in a given leaf and what triggers the break in homeostasis, thus causing Na^+ levels to climb to toxic levels (Figure 6.2)? Some factors worth examination could include subtle changes in water relations, ion build up in the apoplast, or the relative contributions of sheath storage and Na^+ retranslocation. As

discussed in Chapter 4, effective compartmentation as Na^+ gradually accumulates in the leaf in the leaf may 'buy time' for a period of days to weeks for continued optimal rates of photosynthesis. Similar gains could be made if Na^+ homeostasis at non-toxic levels in the leaf is maintained for longer.

6.6.4 Germplasm development and trait evaluation

Further developments from the research completed in this thesis will need to include an evaluation of candidate tissue tolerant and osmotic stress tolerant selections in the field, although this may be complicated if there are large differences in phenology.

Salt tolerance of genotypes will need to be confirmed in the field and genotypes will need to be crossed into adapted local varieties. A number of germplasm development strategies are available. If putative osmotic stress tolerance was confined in released international durum varieties, one or two crosses with the best local variety may suffice to obtain lines appropriate for evaluation. Tissue tolerance was found only in durum related landraces and therefore a backcrossing program with recurrent selection will be necessary to combine this trait with necessary agronomic and grain quality characters. Three to four backcrosses may be required depending on recombination frequency. The success of this approach will depend heavily on the ability to confidently select the tolerant phenotype in a segregating population. Trait evaluation in the field would ideally involve a comparison using isogenic or near-isogenic lines varying for the tolerance phenotype but with identical height, flowering and maturity times.

Field sites for yield evaluation should be chosen with different types and severity of salinity and site characterization would be necessary to account not only for heterogeneity of salinity, but for other abiotic and biotic factors that may influence yield.

6.5 Conclusions

The growth of salt-stressed crop plants such as wheat and barley is limited mostly by the osmotic effects of salinity. Additional limitations on growth occur later, with reduced supply of photo-assimilate to growing tissues due to the accumulation of excessive concentrations of salts in the mature leaves, which results in leaf injury and death. Salt tolerance in wheat has previously been explored, on a mechanistic basis by searching for variation in Na^+ exclusion. Research in this thesis has highlighted the

potential for additional new sources of salt tolerance in durum wheat; osmotic stress tolerance and tissue tolerance. A number of tetraploid landraces and durum varieties have been identified that could be used as novel sources of salt tolerance in wheat breeding programs. Understanding physiological mechanisms at the whole plant level and the cellular level has been crucial for identifying genotypic variation in salt tolerance traits, and will be critical for the further development of quick and reliable phenotypic screening protocols and the introduction of these traits into current durum cultivars and breeding lines.

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8. APPENDICES

Appendix 2.1

T. turgidum landraces (from the Australian Winter Cereals Collection), used in tissue tolerance screen (Experiment 3, Chapter 2).

CSIRO seed catalog no.	Common name	Description	*T.turgidum* sub-species	Line no.	AUS #
P00010	LY/2/6/3		durum	110	-
P00011	LY/3/2/1		durum	111	-
P00012	Graza		durum	112	297
P00013	Mummy	ramified	turgidum	313	3063
P00014	Khans Thesis		carthlicum	414	3549
P00015	-		carthlicum	415	3549
P00016	-		polonicum	216	3824
P00017	-		polonicum	217	3826
P00018	-		carthlicum	418	3829
P00019	-		carthlicum	419	3829
P00020	-		carthlicum	420	3830
P00021	-		carthlicum	421	3832
P00022	-		carthlicum	422	3835
P00023	-		carthlicum	423	3838
P00025	Abyssinia25		polonicum	225	4049
P00026	Crete4		polonicum	226	4280
P00027	Iraq20		polonicum	227	4901
P00028	Portugal170		turanicum	528	5523
P00029	Portugal180		polonicum	229	5533
P00030	Athni		durum	130	6246
P00031	Dandan-1-Shutur		turanicum	531	7810
P00032	Duro		durum	132	7812
P00033	Duro		turgidum	333	7812
P00034	835		durum	134	7815
P00035	835		durum	135	7815
P00036	2843	waxless	durum	136	7829
P00037	2882		durum	137	7842
P00038	Francesone		durum	138	7922
P00039	Beyas		durum	139	8035
P00040	C17875		carthlicum	440	12803
P00041	GranosBlancos		durum	141	12818
P00042	Gandum-I-Shutur		turanicum	542	13538
P00043	Gandum-I-Shutur		turanicum	543	13539
P00044	anon		turanicum	544	14210
P00045	Misr-Bugdaj		turanicum	545	15198
P00046	NicosiaAR1.7.13		durum	146	16014
P00047	NicosiaAR1.7.13		durum	147	16014
P00048	Koko		polonicum	248	16133
P00049	anon		durum	149	17045
P00050	anon	waxless	durum	150	17050
P00051	anon		durum	151	17051
P00052	Quilafen	dwarf	durum	152	17294
P00053	Misr-Bugdaj		turgidum	353	17647
P00054	CudesnajaBlagodat	ramified	turgidum	354	17648
P00055	Bari7418		polonicum	255	18231
P00056	Gigantesingles		durum	156	20677
P00057	Gigantesingles		durum	157	20677
P00058	Wonderling	ramified	turgidum	358	21228
P00059	-		durum	159	22300
P00060	-		durum	160	22303
P00061	-		durum	161	22303
P00062	-	ramified	turgidum	362	22307
P00063	-	ramified	turgidum	363	22307
P00064	-		polonicum	264	22342
P00065	-		polonicum	265	22342
P00066	-		polonicum	266	22345
P00067	-		polonicum	267	22473

Appendix 2.2

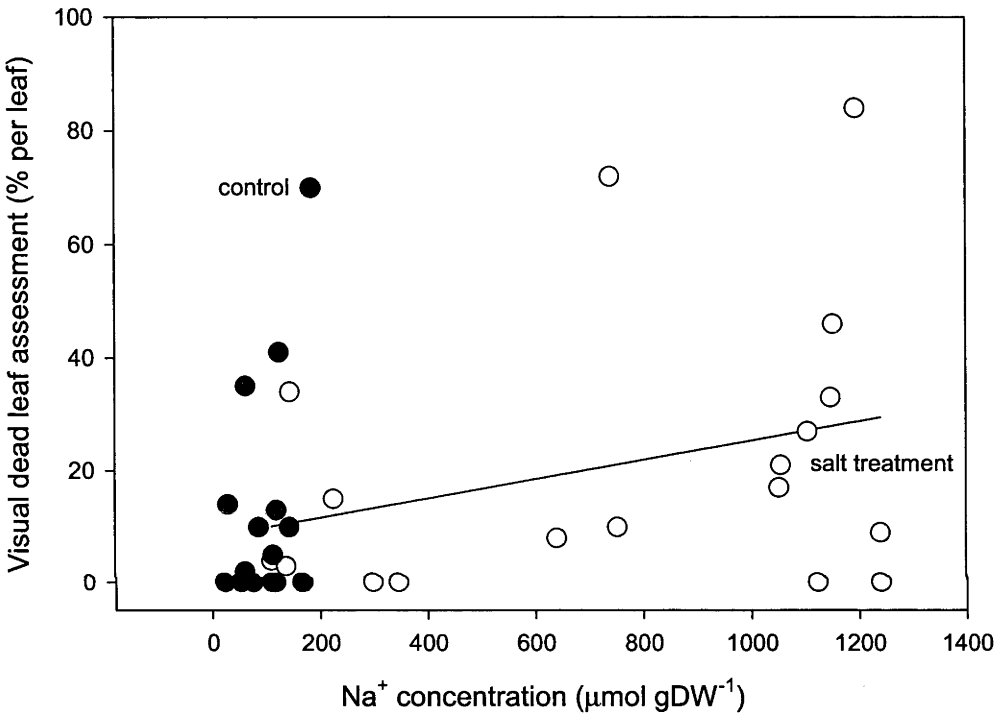
Modified Hoagland's Nutrient Solution (for hydroponics / gravel culture)

Stock solution	Compound	MW	Final concentration	Stock (x125) Amount per 1L (g)	Stock (x1000) Amount per 1L (g)	Use (ml / L)
A	KNO_3	101.11	6.5 mM	82.15	-	8
	$Ca(NO_3)_2.4H_2O$	236.16	4.0 mM	118.08	-	
B	$NH_4H_2PO_4$	115.03	100 µM	1.44	-	8
	$MgSO_4.7H_2O$	246.47	2.0mM	61.62	-	
C	H_3BO_3	61.83	4.6 µM	-	0.284	1
	$MnCl_2.4H_2O$	197.9	0.5 µM	-	0.099	
	$ZnSO_4.7H_2O$	287.54	0.2 µM	-	0.055	
	$(NH_4)_6Mo_7O_{24}.4H_2O$	1235.95	0.1 µM	-	0.124	
	$CuSO_4.5H_2O$	249.7	0.2 µM	-	0.050	
D	$FeCl_3$	162.2	45 µM	24 (ml)		1
	Parts: (in 1L)					
	NaOH			5.0		
	EDTA-Na			32.2		
	$FeCl_3$	162.2	45 µM	24 (ml)		

Note: Na^+ concentration in ½ modified Hoagland's Solution is 0.1 mM

Appendix 2.3

Relationship between leaf Na^+ concentration and leaf injury assessed visually in leaves from control (●) and salt-treated (○) tetraploid seedlings grown in control or 150 mM NaCl for 17 d. Values for dead leaf assessment are means (n = 9). The linear regression fitted to the salt treatment data is described by the following equations: y = 0.017x + 8.217 (r^2 = 0.09).



Appendix 2.4

(% DL), the ratio of % DL to total leaf Na^+, Na^+ concentration in dead leaf and mean chlorophyll estimate (leaves 1, 2 and 3 on main stem) in different subspecies of *T. turgidum*, durum wheat cultivars, bread wheat cultivars and a barley cultivar grown in 150 mM NaCl for 21 d.

Sub-species	Line	%DL (%)	Ratio of %DL to leaf Na^+ content (mmol)	Na^+ concentration in dead leaf (mmol gDW^{-1})	Mean chlorophyll content of leaves 1, 2 & 3 (SPAD units)
Ssp. durum	112	8.7	27	2.28	19.8
	130	15.2	35	4.69	15.7
	132	9.4	25	4.36	19.8
	135	8.9	19	4.10	20.9
	136	10.4	29	4.61	16.7
	137	8.9	17	4.25	23.2
	138	11.2	26	4.43	24.0
	139	5.1	24	3.95	30.8
	141	12.4	25	3.59	12.2
	147	11.8	36	3.57	18.0
	149	5.1	32	2.38	35.1
	150	13.3	67	4.28	21.3
	151	9.6	36	3.83	25.3
	152	9.4	17	3.29	17.4
	156	6.4	21	4.45	31.5
	159	10.9	22	3.76	24.3
	161	15.0	33	1.46	18.9
Ssp. polonicum	216	5.7	12	3.90	23.8
	217	8.3	20	4.35	18.6
	225	8.0	19	5.11	19.2
	226	7.9	15	4.45	19.1
	227	9.4	18	4.21	18.1
	229	9.2	21	4.32	17.3
	248	8.6	18	3.98	20.6
	255	4.2	11	3.81	31.5
	264	5.8	15	3.81	24.8
	266	10.3	20	4.70	18.1
	267	8.4	15	4.57	19.1
Ssp. turgidum	313	11.1	22	4.03	20.5
	333	6.1	13	4.32	25.1
	353	12.5	36	3.68	21.6
	354	8.4	17	4.08	23.4
	358	12.0	27	4.16	16.0
	362	5.6	11	3.69	29.9
	363	7.1	14	3.60	25.8
Ssp. carthlicum	414	2.2	9	3.87	32.7
	419	44.7	59	3.52	5.1
	420	26.4	57	3.39	21.2
	421	16.5	38	2.97	19.3
	422	28.4	51	3.66	10.8
	423	23.2	59	3.10	18.8
	440	11.9	41	4.23	17.9
Ssp. turanicum	528	6.0	15	2.52	36.6
	531	13.2	35	3.49	20.7
	542	12.0	37	3.42	20.1
	543	9.0	35	3.25	27.9
	544	8.9	21	3.47	22.0
	545	11.4	35	3.69	19.7

Appendix 2.5

Na^+, K^+ and Cl^- concentrations (mM), corresponding osmotic potentials (bars), and leaf osmotic potential (π) of two durum cultivars, a barley cultivar and six tetraploid selections. All measurements were on leaf 3 after 10 d in 150 mM NaCl .

Genotype	Na^+		K^+		Cl^-		Na^++K^++Cl^-	π
	(mM)	(bars)	(mM)	(bars)	(mM)	(bars)	(bars)	(bars)
Durum cultivars:								
Wollaroi	196	-4.90	142	-3.55	284	-7.10	-15.55	-21.0
Tamaroi	206	-5.15	165	-4.13	285	-7.13	-16.41	-19.1
Barley cultivar:								
Skiff	242	-6.05	106	-2.65	206	-5.15	-13.85	-19.1
Tetraploid lines:								
Line 362	244	-6.10	140	-3.50	213	-5.33	-14.93	-20.9
Line 255	220	-5.50	145	-3.63	191	-4.78	-13.91	-20.7
Line 139	243	-6.08	145	-3.63	201	-5.03	-14.74	-21.0
Line 528	235	-5.88	151	-3.78	184	-4.60	-14.26	-18.6
Line 414	240	-6.00	143	-3.57	191	-4.77	-14.34	-21.0
Low Na+ line:								
Line 149	47	-1.18	290	-7.25	182	-4.55	-12.98	-18.3

Appendix 5.1

Durum varieties and *T. turgidum* landraces (from the Australian Winter Cereals Collection), bread wheats and barley used in stomatal conductance screens (Experiments 1 – 3; Chapter 5).

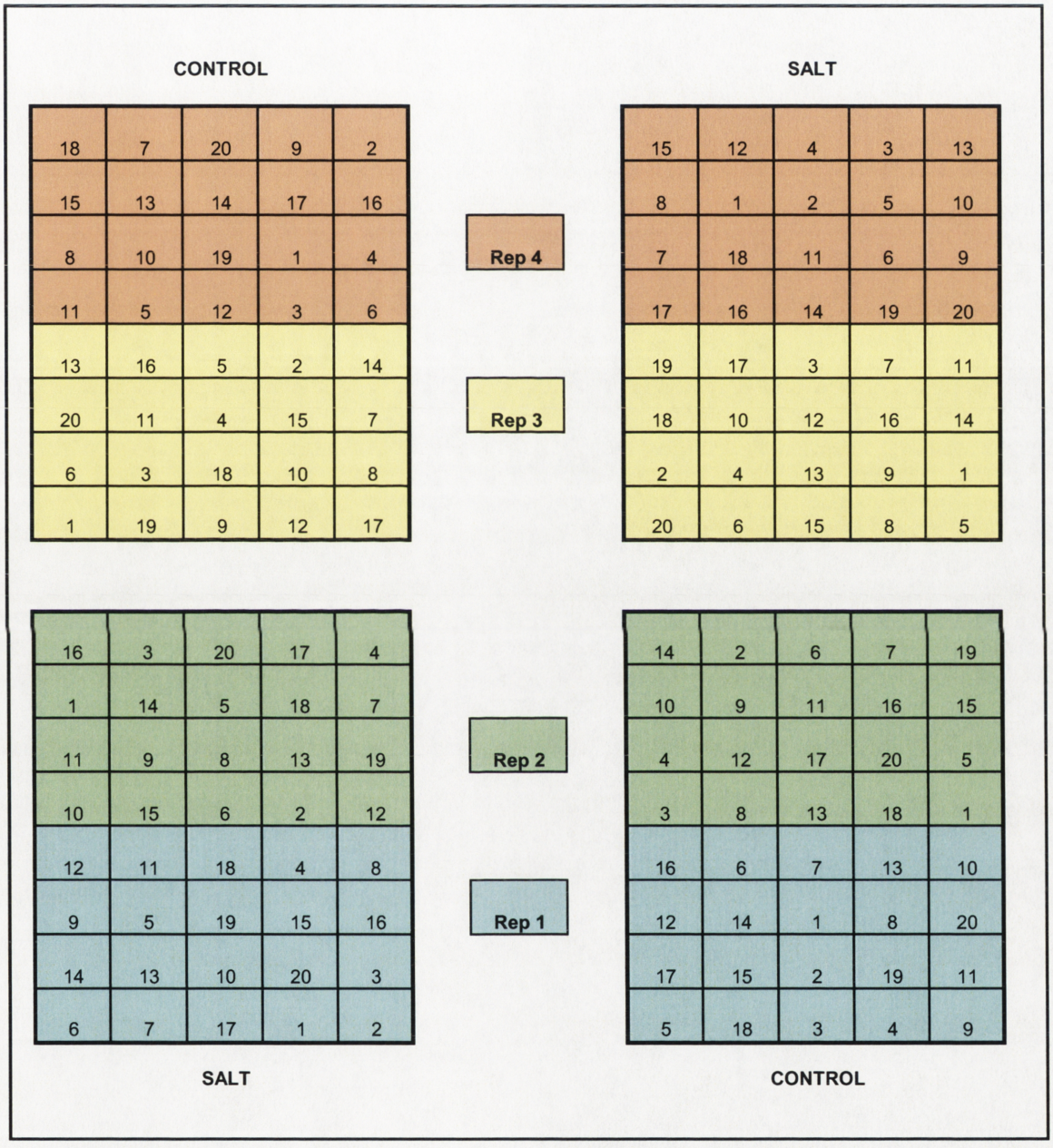
Exp.	Category	Description	Variety/ genotype name	AUS #
1	Checks	Barley cv.	Franklin	405994
		Hexaploid landrace	Kharchia	20741
		Australian bread wheat cv.	Westonia	30128
		Australian durum cv.	Bellaroi	30781
		Australian durum cv.	Tamaroi	27663
		Australian durum cv.	Wollaroi	25926
	Putative tissue tolerant lines	ssp. *carthlicum*	Line 414	3549
	(*T.turgidum* landraces, Chapter 2)	ssp. *turanicum*	Line 528	5523
		ssp. *durum*	Line 139	8035
		ssp. *polonicum*	Line 255	18231
		ssp. *turgidum*	Line 362	22307
	Breeding line (Ray Hare)		BL950090	-
	Australian Winter Cereals Collection		Khabur	-
			Solid stem durum	1260
			Azul de Carmona Linea 291	7875
			Brachowa	23840
			Durex	27170
			Dahutia	10097
			Nordgau	21976
2	Checks	Australian durum cv.	Tamaroi	27663
		Hexaploid landrace	Kharchia	20741
	Breeding line (Ray Hare)		BL 970023	-
	Australian Winter Cereals Collection		Bouffarick	8611
			Brkulja	8919
			Emblem	9472
			Langlais	2844
			AC Morse	27171
			Seklavi	8899
			Coulter	19805
			Hercules	10662
			Vic	20713
			Volga W1515	26717
			Turkestan W7450	26694
			Edmore	22137
			Koelz W3158	15337
			Candicans	9857
			Guarani	8634
			Garigliano	12380
			Persia 16	26560

Appendix 5.1 (cont.)

Exp.	Category	Description	Variety/ genotype name	AUS #
3	*T.turgidum* landraces, Chapter 2	ssp. *polonicum*	Line 264	22342
			Line 229	5533
			Line 226	4280
			Line 216	3824
		ssp. *durum*	Line 156	20677
			Line 152	17294
			Line 146	16014
			Line 141	12818
			Line 138	7922
			Line 137	7842
			Line 132	7812
			Line 130	6246
		ssp. *carthlicum*	Line 440	12803
			Line 420	3830
			Line 415	3549
			Line 414	3549
	Australian Winter Cereals Collection Checks		Icaro	25287
		Australian durum cv.	Kalka	33876
		Australian durum cv.	Wollaroi	25926
		Australian durum cv.	Tamaroi	27663

Appendix 5.2

Latinized spatial random experimental design used in stomatal conductance screens (Experiments 1 – 4). Numbers 1 – 20 represent 20 individual lines, replicated 4 times in control (non-saline) and salt (150 mM NaCl) treatments.



Appendix 5.3

Stomatal conductance data for lines in Experiments 1 – 3 (Chapter 5).

Exp.	Name	Stomatal conductance (mmol m^{-2} s^{-1})		Corrected and transformed data		
		Control	Salt	Control	Salt	Salt (% control)
		Mean ± SE	Mean ± SE			
1	Franklin	448 ± 45	421 ± 107	21.70	20.37	93.9
	Kharchia	332 ± 95	148 ± 37	17.98	11.58	64.4
	Westonia	870 ± 55	266 ± 98	28.48	14.61	51.3
	Bellaroi	552 ± 159	326 ± 99	23.15	18.81	81.3
	Tamaroi	765 ± 89	202 ± 61	27.07	14.04	51.9
	Wollaroi	853 ± 97	313 ± 73	24.71	14.96	60.5
	Line 414	103 ± 16	62 ± 26	10.38	7.42	71.5
	Line 528	480 ± 23	300 ± 64	21.64	16.02	74.0
	Line 139	227 ± 88	132 ± 39	13.51	10.71	79.3
	Line 255	155 ± 38	75 ± 13	10.73	8.32	77.5
	Line 362	492 ± 70	270 ± 76	21.59	15.85	73.4
	BL950090	571 ± 123	159 ± 60	22.40	11.67	52.1
	Khabur	420 ± 50	240 ± 68	20.25	13.68	67.6
	Solid stem durum	209 ± 103	64 ± 47	11.25	6.39	56.8
	Azul de Carmona Linea 291	701 ± 144	430 ± 131	26.15	19.62	75.0
	Brachowa	618 ± 116	359 ± 111	25.29	17.27	68.3
	Durex	723 ± 152	93 ± 11	25.85	9.94	38.5
	Dahutia	772 ± 101	385 ± 114	26.98	19.40	71.9
	Nordgau	605 ± 148	186 ± 40	23.01	13.97	60.7
2	Tamaroi	570 ± 80	259 ± 39	23.57	15.55	66.0
	Kharchia	197 ± 44	81 ± 19	14.69	8.51	57.9
	BL 970023	326 ± 65	67 ± 10	22.29	11.29	50.7
	Brkulja	481 ± 87	141 ± 37	16.85	8.19	48.6
	Emblem	408 ± 48	353 ± 32	18.67	18.78	100.6
	Langlais	655 ± 62	420 ± 31	25.65	20.45	79.7
	AC Morse	566 ± 97	330 ± 35	24.65	18.05	73.2
	Seklavi	375 ± 65	423 ± 94	19.5	20.57	105.5
	Coulter	780 ± 41	665 ± 43	27.7	25.28	91.3
	Hercules	765 ± 93	577 ± 51	27.78	23.27	83.8
	Vic	566 ± 53	405 ± 61	23.44	20.58	87.8
	Volga W1515	227 ± 71	185 ± 47	13.78	13.64	99.0
	Turkestan W7450	230 ± 80	118 ± 13	14.51	10.84	74.7
	Edmore	539 ± 114	408 ± 126	22.37	23.78	106.3
	Koelz W3158	528 ± 134	175± 23	22.13	12.62	57.0
	Candicans	316 ± 100	52 ± 9	16.42	6.74	41.0
	Guarani	543 ± 131	273 ± 36	22.74	16.36	71.9
	Garigliano	715 ± 132	501 ± 25	25.82	22.44	86.9
	Persia 16	723 ± 39	373 ± 57	27.08	19.59	72.3
3	Line 264	446 ± 70	89 ± 5	20.93	9.41	45.0
	Line 229	479 ± 51	103 ± 19	21.78	10.03	46.1
	Line 226	564 ± 106	86 ± 13	23.42	9.19	39.2
	Line 216	517 ± 51	86 ± 16	22.9	9.12	39.8
	Line 156	544 ± 27	105 ± 28	23.3	9.92	42.6
	Line 152	648 ± 15	177 ± 40	25.44	12.94	50.9
	Line 146	518 ± 44	131 ± 39	22.68	11.1	48.9
	Line 141	521 ± 29	238 ± 36	22.8	15.27	67.0
	Line 138	445 ± 19	56 ± 9	21.08	7.44	35.3
	Line 137	560 ± 37	182 ± 36	23.63	13.26	56.1
	Line 132	728 ± 68	217 ± 18	26.88	14.69	54.7
	Line 130	386 ± 32	150 ± 16	19.6	12.19	62.2
	Line 440	533 ± 70	147 ± 20	22.94	12.04	52.5
	Line 420	471 ± 40	181 ± 17	21.65	13.42	62.0
	Line 415	423 ± 58	78 ± 13	20.41	8.75	42.9
	Line 414	401 ± 42	66 ± 12	19.95	8.03	40.3
	Icaro	509 ± 45	110 ± 11	22.49	10.44	46.4
	Kalka	348 ± 27	123 ± 14	18.87	11.03	58.5
	Wollaroi	576 ± 93	100 ± 20	23.75	9.82	41.3
	Tamaroi	568 ± 30	120 ± 31	23.8	10.67	44.8